

**Modulation of sarcoplasmic reticulum gene expression due to  
ischemia-reperfusion in isolated rat heart**

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**By**

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**Modulation of Sarcoplasmic Reticulum Gene Expression due to  
Ischemia-Reperfusion in Isolated Rat Heart**

**BY**

**Rana M. Temsah**

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

**of**

**Doctor of Philosophy**

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**Dedicated to**

**The people who inspired me**

**My dad, mom, my brother Hani,**

**my sister Rima and her family: Fuad,**

**the twin angels Mohammad and Mustapha**

**and to the newly born angel**

**Ali**

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## ABSTRACT

Ischemia-reperfusion (IR) is a common feature present in different pathologies such as acute myocardial infarction and ischemic heart disease as well as in various clinical maneuvers following cardiac bypass surgery, heart transplantation, coronary angioplasty and thrombolysis. In view of the fact that the sarcoplasmic reticulum (SR) is intimately involved in the cardiac contraction-relaxation cycle and is known to regulate the intracellular concentration of  $\text{Ca}^{2+}$ , defects in SR  $\text{Ca}^{2+}$ -pump ATPase and  $\text{Ca}^{2+}$ -release channels have been identified to explain  $\text{Ca}^{2+}$ -handling abnormalities and subsequent cardiac dysfunction in IR hearts. This study addressed the following hypothesis: IR induces long-term cardiac dysfunction in remnant myocytes by modulating SR mRNA expression for the  $\text{Ca}^{2+}$ -cycling proteins such as  $\text{Ca}^{2+}$ -pump ATPase,  $\text{Ca}^{2+}$ -release channels, phospholamban and calsequestrin. The objectives of this investigation were to examine: a) the effect of IR on the SR mRNA and protein expression, b) the effect of hypoxia and glucose deprivation that occur during ischemia on SR function, c) the role of oxidative stress and  $\text{Ca}^{2+}$ -overload in the IR-induced SR changes, and d) the effects of antioxidants,  $\beta$ -adrenoceptor blockers and ischemic-preconditioning in preventing IR-induced changes in SR.

Thirty minutes of global ischemia followed by 60 min of reperfusion of the isolated rat heart resulted in impaired cardiac performance, SR function as well as reduced levels of SR  $\text{Ca}^{2+}$ -cycling proteins. These changes were accompanied by depressed mRNA expression of SR proteins. In view of the occurrence of hypoxia

and substrate lack in the ischemic heart, we investigated the role of these factors in the alterations observed during reperfusion. Hearts perfused with oxygenated medium containing glucose after 30 min of oxygen or glucose deprivation showed partial recovery of heart function with normal levels of expression of the SR mRNA and proteins. Hearts perfused with normal medium after deprivation of both glucose and oxygen showed partial functional recovery with normal levels of SR protein expression despite depressed transcript levels of the SR  $\text{Ca}^{2+}$ -cycling proteins. This implicates that both hypoxia and glucose deprivation during the ischemic phase are important factors for the occurrence of IR-induced injury.

The role of oxidative stress in IR induced injury was investigated by treating IR hearts with superoxide dismutase plus catalase. This treatment improved cardiac performance, SR function and mRNA expression in the IR hearts. Alterations provoked due to oxidative stress were confirmed when hearts treated with  $\text{H}_2\text{O}_2$  and xanthine plus xanthine oxidase (source of superoxide radical) showed results similar to those observed in the IR hearts. Some experiments were also carried out to examine the effect of  $\text{Ca}^{2+}$ -overload, which occurs during the reperfusion phase of IR, on cardiac performance and SR function. For this purpose,  $\text{Ca}^{2+}$ -overload was induced by perfusing hearts for 5 min with a  $\text{Ca}^{2+}$ -free buffer followed by 30 min of perfusion with a  $\text{Ca}^{2+}$ -containing medium.  $\text{Ca}^{2+}$ -depleted/repleted hearts showed impaired functional recovery with drastic decrease in the SR function, protein content and mRNA expression. These results indicate that both oxidative stress and  $\text{Ca}^{2+}$ -overload are

major players in inducing changes in SR function and mRNA expression in the IR hearts.

IR hearts treated with  $\beta$ -adrenoceptor blockers (atenolol or propranolol) have shown marked protection of cardiac performance, SR function and protein and mRNA expression of the SR proteins. Similar results were also observed when the IR maneuver was preceded by three cycles of brief episodes of ischemia and reperfusion (ischemic-preconditioning). These results suggest that the activation of the adrenergic system in IR hearts may contribute towards the occurrence of IR-induced changes in SR function whereas ischemic preconditioning may exert beneficial effect by attenuating the IR-induced changes in SR mRNA expression.

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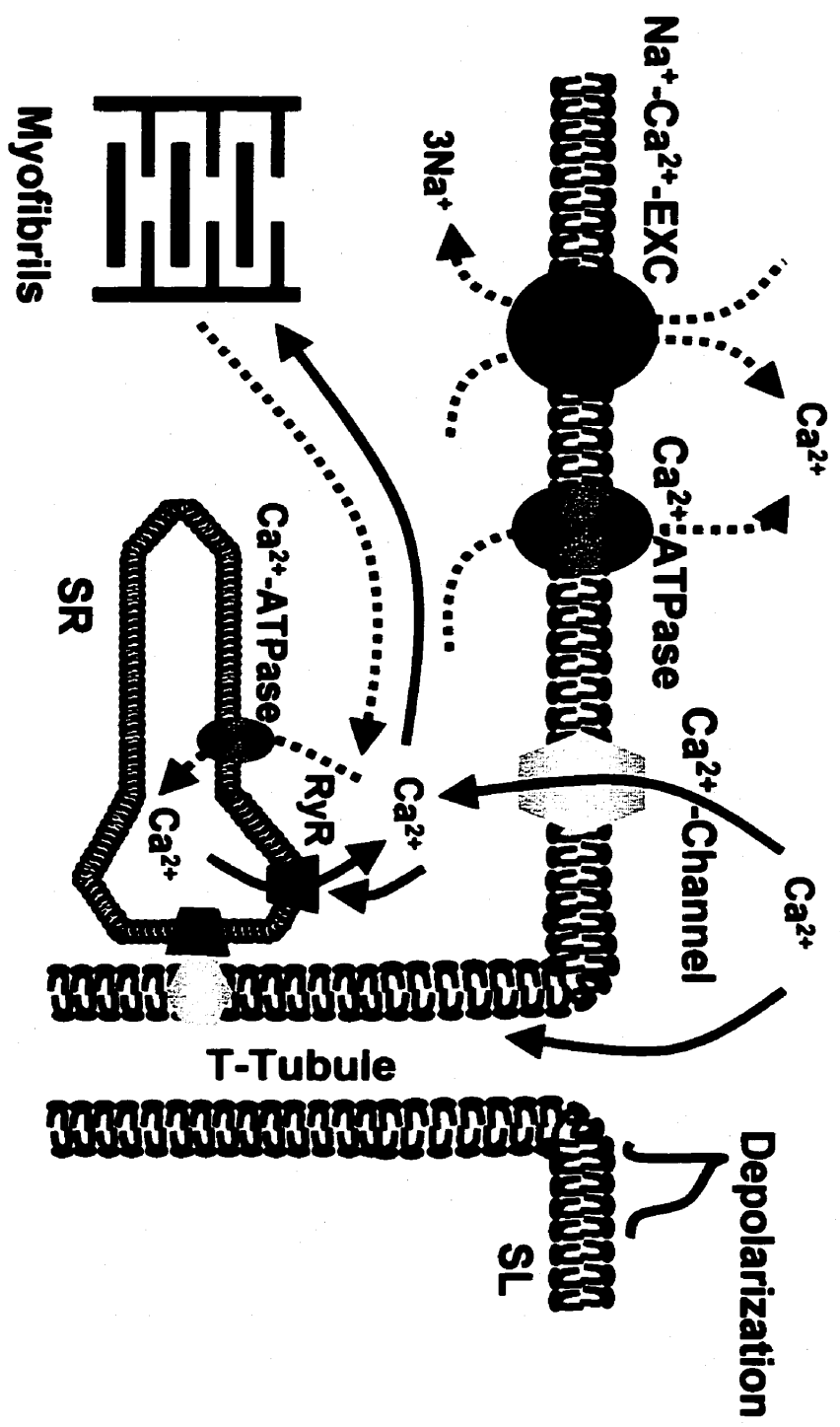
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## I. LITERATURE REVIEW

It is well established that calcium is an essential cation for the maintenance of cellular integrity, regulation of metabolism, cell growth and cell proliferation (1). This cation plays a critical role in cardiomyocyte function on a beat-to-beat basis as it is involved in the process of excitation-contraction coupling and relaxation. A large gradient of  $\text{Ca}^{2+}$  (about 10,000 fold) across the cardiomyocytes membrane is maintained due to the coordinated function of different cellular organelles such as sarcolemma (SL), sarcoplasmic reticulum (SR) and mitochondria. The concentration of ionized  $\text{Ca}^{2+}$  in the extracellular space is about 1.25 mM whereas the intracellular  $\text{Ca}^{2+}$  concentration ranges between  $10^{-7}$  M and  $10^{-5}$  M during diastole and systole, respectively. In view of the major role of SR in the regulation of intracellular  $\text{Ca}^{2+}$  in cardiomyocytes, this review of literature is focused on discussing cardiac SR function in health and disease with particular attention to problems associated with ischemia-reperfusion (IR) injury and heart failure.

### 1. Physiological and biochemical aspects of SR system

In cardiomyocytes, both SL and SR are considered to be the major organelles involved in the regulation of intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) on a beat-to-beat basis (**Figure 1**). Depolarization of the SL membrane permits entry of a relatively small amount of  $\text{Ca}^{2+}$  from the extracellular space (primary source of  $\text{Ca}^{2+}$ ) via L-type  $\text{Ca}^{2+}$ -channels (2). This slight rise in  $[\text{Ca}^{2+}]_i$  triggers the release of



**Figure 1:** Schematic representation of Ca<sup>2+</sup>-movements in myocardial cell. SL, sarcolemma; SR, sarcoplasmic reticulum; T-tubule, transverse tubule; Na<sup>+</sup>-Ca<sup>2+</sup>-EXC, Na<sup>+</sup>-Ca<sup>2+</sup>-exchanger; Ca<sup>2+</sup>-ATPase, Ca<sup>2+</sup>-pump ATPase; RyR, ryanodine receptor. Straight arrows indicate the mechanisms that increase the cytoplasmic Ca<sup>2+</sup> to enhance the cross-bridge attachment and dashed arrows indicate the mechanisms that restore the cytoplasmic Ca<sup>2+</sup> level back to normal.

a large amount of  $\text{Ca}^{2+}$  from the SR store through  $\text{Ca}^{2+}$ -release channels; a phenomenon known as  $\text{Ca}^{2+}$ -induced- $\text{Ca}^{2+}$ -release (2, 3). The released  $\text{Ca}^{2+}$  activates the contractile filaments and hence results in cardiac contraction. For the occurrence of cardiac relaxation, the level of  $[\text{Ca}^{2+}]_i$  is restored to the resting level by the activity of the SR  $\text{Ca}^{2+}$ -pump ATPase (4, 5), exchange of  $\text{Ca}^{2+}$  for  $\text{Na}^+$  via the  $\text{Na}^+$ - $\text{Ca}^{2+}$ -exchanger (6, 7) and by SL  $\text{Ca}^{2+}$ -pump ATPase. At this point, it is important to mention that the contribution of these systems is species dependent. Bers et al (6, 8) have shown that in rat ventricular myocardium SR  $\text{Ca}^{2+}$ -pump ATPase is responsible for 92% of  $\text{Ca}^{2+}$  uptake while 7% of  $\text{Ca}^{2+}$  is removed by the  $\text{Na}^+$ - $\text{Ca}^{2+}$ -exchanger. In human, rabbit, cat, ferret and guinea pig the SR  $\text{Ca}^{2+}$ -pump ATPase removes 70-75% of  $\text{Ca}^{2+}$ , whereas 25-30% is removed by  $\text{Na}^+$ - $\text{Ca}^{2+}$ -exchanger. The SL  $\text{Ca}^{2+}$ -pump ATPase has been considered of minor importance. Although nuclei and mitochondria are also known to accumulate a large amount of  $\text{Ca}^{2+}$ , their role in the regulation of intracellular  $\text{Ca}^{2+}$  in cardiomyocytes is not fully defined.

Ultrastructural studies of ventricular myocardial cells have revealed that the SR is composed of at least three distinct structures (9): a) network SR which represents the major region of the SR surrounding the myofibrils (10), b) peripheral SR and interior-junctional SR that are closely opposed to the SL and T-tubules, respectively; these are composed of cisternae and longitudinal regions where the former is connected by junctional processes called "feet" (10, 11), and c) corbular SR that is confined to the I-band of the sarcomere. Both the junctional

SR and corbular SR are extensions of the network SR. The SR membranes are composed of several proteins that are of functional significance among which are:  $\text{Ca}^{2+}$ -release channels or ryanodine receptors (RyR),  $\text{Ca}^{2+}$ -pump ATPase (SERCA2a), phospholamban (PLB) and calsequestrin (CQS).

Cardiac contraction is triggered by the release of  $\text{Ca}^{2+}$  from the SR by  $\text{Ca}^{2+}$ -release channels, also referred to as RyR. The channel acquired this nomenclature from its capability to bind to ryanodine which is a highly toxic plant alkaloid and induces different effects depending on the doses applied (12). Nanomolar concentrations are known to keep the channel in an open state, whereas concentrations higher than 10  $\mu\text{M}$  can completely close the channel. In cardiomyocytes, two different  $\text{Ca}^{2+}$ -release channels have been identified: RyR which is most abundant and relevant to excitation-contraction coupling; and the inositol 3, 4, 5-triphosphate receptor which is of low density in the SR (13). Using molecular cloning analysis, RyR was detected in three different isoforms (RyR1, RyR2 and RyR3) encoded by three different genes. RyR2 is the only isoform expressed in cardiac tissue (14-16) and is considered the largest protein identified in the SR (565 kDa) (15). It consists of over 5,000 amino acids (17, 18) and is composed of four monomers that form a tetrameric structure. RyR forms a functional complex due to its association with several other proteins: CQS, junctin, triadin and FK506 binding protein (KFBP) (19). Stoke and Wagenknecht (20) have suggested that the unusual high molecular mass of RyR may be due to numerous endogenous modulatory ligands such as  $\text{Ca}^{2+}$ , calmodulin, nitric oxide,



L-type  $\text{Ca}^{2+}$ -channel and an associated protein known as FKBP binding protein (13). RyR is also known to be phosphorylated by  $\text{Ca}^{2+}$ /calmodulin dependent protein kinase (CaMK) at Ser-2809 and by cAMP-dependent protein kinase (PKA) (15, 21, 22). Anatomically, the proximity between the L-type  $\text{Ca}^{2+}$ -channels on the T-tubules and RyR at the cisternae of the SR allows what is known as the  $\text{Ca}^{2+}$ -induced- $\text{Ca}^{2+}$ -release phenomenon (2).

The sarco(endo)plasmic reticulum  $\text{Ca}^{2+}$ -pump ATPase (SERCA) is encoded by three highly homologous genes: SERCA1, SERCA2, and SERCA3 (23). SERCA1a and 1b isoforms are expressed in adult and neonatal fast-twitch skeletal muscles, respectively (24). SERCA2a is the cardiac and slow-twitch skeletal isoform (25), whereas SERCA2b is expressed in smooth muscle and non-muscle tissues (26). SERCA3 is a non-muscle isoform and it is mainly expressed in epithelial and endothelial cells (27). It is now well established that SERCA2a is the only isoform expressed in normal or diseased myocardium (28-30). The pump (105 kDa), a  $\text{Ca}^{2+}$ -stimulated and  $\text{Mg}^{2+}$ -dependent ATPase protein (31,32), is localized mainly in the longitudinal portion of the SR (33) and constitutes 35-40% of the SR proteins (34, 35). By the hydrolysis of one molecule of ATP,  $\text{Ca}^{2+}$ -pump transports two  $\text{Ca}^{2+}$ -ions against a high ionic gradient ranging between 100 nM - 10  $\mu\text{M}$  in the cytosol and 1 mM in the SR (8). Since SERCA2a activity determines the amount of sequestered  $\text{Ca}^{2+}$  to be available for release in the next wave of excitation and is responsible for the restoration of  $\text{Ca}^{2+}$  gradient between intracellular and SR lumen side, SERCA2a is an essential protein for the

determination of the rate and extent of relaxation and the rate and amplitude of contraction. SERCA2a is also known to undergo direct phosphorylation by CaMK at Ser-38 (36) resulting in enhanced  $\text{Ca}^{2+}$ -uptake in the SR.

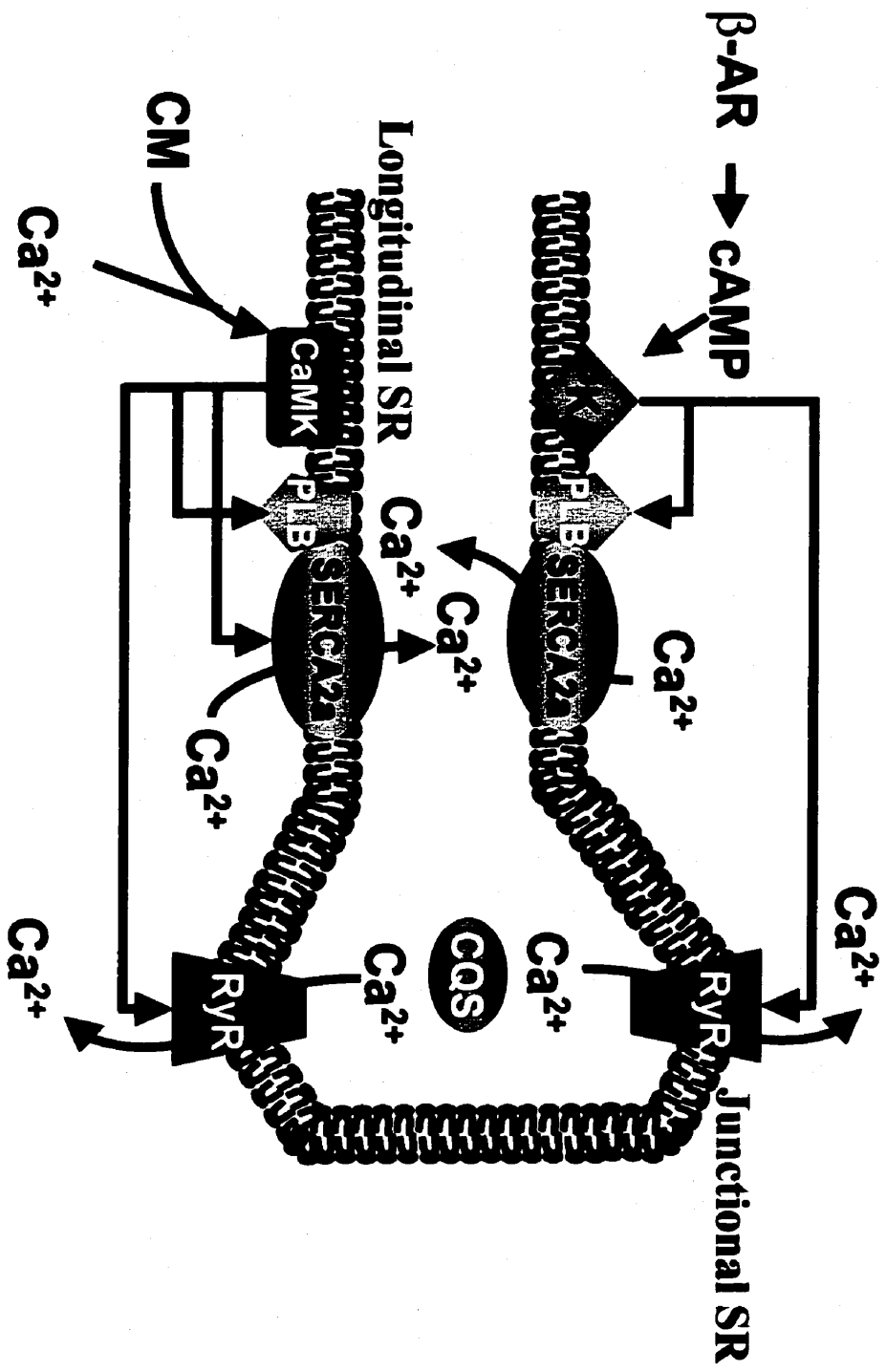
PLB is a regulatory phosphoprotein in the SR membrane that is remarkably conserved since it is encoded by one gene in all species and is expressed in one form in the cardiac and skeletal muscle (37). Cardiac PLB is comprised of two low molecular weight forms of proteins, the pentameric form and the monomeric form (38-40). The pentameric form (27 kDa) is composed of 5 identical subunits which upon boiling in sodium dodecyl sulfate buffer dissociates into identical monomers (6 kDa) having 52 amino acids (41-43). Since the pentameric and monomeric forms of PLB are under dynamic equilibrium, it is suggested that the pentameric form may be an inactive reservoir for the active monomeric form (44). Immunohistochemical studies have shown that PLB is localized in the SR with the highest distribution in the longitudinal region of the SR and co-localized with SERCA2a. This anatomical proximity indicates the functional correlation between both proteins. PLB has been identified as the principal substrate phosphorylated by PKA at Ser-16, CaMK at Thr-17, protein kinase C at Ser-10 and by cGMP-dependent protein kinase (22, 42, 45-47). PLB phosphorylation *in vivo* has been postulated to play a key role in mediating a lusitropic effect (cardiac relaxation) and the inotropic effect (cardiac contractility) of the  $\beta$ -adrenergic system (45).

Calsequestrin (CQS) is characterized as a high-capacity moderate affinity  $\text{Ca}^{2+}$ -binding protein which stores  $\text{Ca}^{2+}$  in the SR lumen (48-50) for release by the

next wave of depolarization. Among the two isoforms of CQS only one isoform is expressed in the developing, adult and aging cardiac tissue (51-53). CQS (55 kDa) is composed of 396 amino acid residues and is anchored to the cisternae part of the SR in close proximity with RyR (54). CQS, RyR and other SR proteins (FKBP, junctin and triadin) are hypothesized to form a functional complex for the coordination of  $\text{Ca}^{2+}$ -release (19). Although CQS is known to be a preferred substrate for phosphorylation by casein kinase II at Ser-378 both *in vivo* and *in vitro* (55), the functional consequence of this phosphorylation is not yet understood.

## 2. Regulation of SR function by phosphorylation

There is an increasing body of evidence suggesting that  $\text{Ca}^{2+}$  flux during cardiac contraction-relaxation cycle are controlled by phosphorylation and dephosphorylation of the proteins involved in  $\text{Ca}^{2+}$ -movements. The balance between these two processes is important in modulating cellular responses to different stimuli by regulating  $\text{Ca}^{2+}$ -homeostasis in cardiomyocytes. It is now well established that the SR function is regulated by an endogenous CaMK (56) and PKA (57) mediated phosphorylation of SR proteins (**Figure 2**). CaMK phosphorylates RyR, SERCA2a, and PLB whereas PKA phosphorylates RyR and PLB (45, 58, 59). During diastole, the affinity of SERCA2a for  $\text{Ca}^{2+}$  is inhibited by its protein-protein interaction on its cytoplasmic and transmembrane domains



**Figure 2:** Schematic representation of the regulation of SR  $\text{Ca}^{2+}$  flux by  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase (CaMK) and cAMP-dependent protein kinase (PKA) pathways.  $\beta$ -AR,  $\beta$ -adrenergic receptor; CM, calmodulin; CQS, calsequestrin; PLB, phospholamban; RyR, ryanodine receptor; SERCA2a, sarco(endo)plasmic reticulum  $\text{Ca}^{2+}$ -pump ATPase.

by the dephosphorylated form of PLB (60-65). Under physiological conditions, PLB phosphorylation is mediated upon CaMK activation and also by increased levels of cAMP due to the activation of the  $\beta$ -adrenergic system (66). PLB phosphorylation by PKA (47, 61, 62) and CaMK (67-69) relieves this inhibition resulting in increased SERCA2a affinity for  $\text{Ca}^{2+}$  (63, 70), enhanced SR  $\text{Ca}^{2+}$ -uptake (71) and improved cardiac relaxation (28, 72). In turn the SR is loaded with  $\text{Ca}^{2+}$  to be released by the arrival of the next wave of depolarization and therefore induce a positive inotropic response (72, 73). In addition to its regulation by PLB, SERCA2a is directly phosphorylated by CaMK (36) which increases ATP hydrolysis and thus stimulates  $\text{Ca}^{2+}$ -transport into the SR lumen by enhancing  $V_{\text{max}}$  (28, 58, 59). Although extracellular  $\text{Ca}^{2+}$  triggers the release of  $\text{Ca}^{2+}$  from SR via RyR, the latter is known to be phosphorylated by CaMK and PKA; a process that promotes SR  $\text{Ca}^{2+}$ -release due to an increase in the duration of the open state of the channel (21, 22, 74). The phosphorylated proteins are later dephosphorylated by an endogenous phosphatase that reverses the phosphorylation effects (57, 75, 76). The endogenous SR phosphatase has the capability for dephosphorylation of both CaMK and PKA phosphorylated substrates in a non-discriminatory fashion (75, 77).

### **3. Changes in SR function and SR mRNA expression in heart failure**

Because of ethical concerns, human studies are limited to end-stage heart failure especially during heart transplantation procedure, when a failing heart is

replaced by a donor heart from which a sample is considered a proper control. Muscle strips or single myocytes isolated from failing human hearts exhibited an impairment in force development (78, 79), slower relaxation (80, 81), prolonged action potential duration (78), reduced capacity to restore the resting levels of  $\text{Ca}^{2+}$  and reduced ATP turnover (80, 82-84). These abnormalities were accredited to impairment of the  $\text{Ca}^{2+}$ -cycling proteins (85) which was evident by the reported decrease in SR  $\text{Ca}^{2+}$ -uptake,  $\text{Ca}^{2+}$ -release and  $\text{Ca}^{2+}$ -pump ATPase activities (86-90). Some studies have suggested that changes in the protein levels of SERCA2a and PLB are excluded as possible factors mediating cardiac abnormalities (91-93). At this point it is important to mention that alterations in the myofilament sensitivity to  $\text{Ca}^{2+}$  is considered as a major contributing factor in cardiac dysfunction due to heart failure (87, 94).

The general perception established from experimental and human studies was that cardiac dysfunction detected in different heart diseases is tightly linked to alterations in  $\text{Ca}^{2+}$ -handling which may be due to reduced levels of SR  $\text{Ca}^{2+}$ -cycling protein reflecting alterations in mRNA expression. Experimental models of hypertrophy have revealed a downregulation in RyR, SERCA2a, PLB and CQS transcript levels (29, 95-99) (Table 1). These changes were attributed to depressed gene expression in the myocytes and not to cell necrosis (100, 101). Moreover, direct gene transfer method revealed that the decrease in the mRNA levels of SERCA2a in failing hearts due to pressure overload is transcriptional in nature (99, 102). Nevertheless, there is evidence available in the literature which

**Table 1: Alterations in mRNA expression and protein content of the SR Ca<sup>2+</sup>-cycling proteins in experimental models.**

Model	Species	SR Ca <sup>2+</sup> -cycling proteins		Functional evidences	Ref.
		mRNA	Protein		
PO (1-weeks)	Rabbit	65% ↓ SERCA2a	ND		29
(4-weeks)		64% ↓ PLB	ND		
		44% ↓ SERCA2a	ND		
		↔ PLB	ND		
PO (16-days)*	Rabbit	92% ↓ SERCA2a	22% ↓ SERCA2a	22% ↓ Ca <sup>2+</sup> -uptake	261
		51% ↓ RyR	ND	19% ↓ phosphoenzyme	
		84% ↓ PLB	ND		
		70% ↓ CQS	ND		
VO (35-days)	Rat	20% ↓ SERCA2a	ND	27% ↑ LVEDP	263
		29% ↓ RyR	ND	12% ↓ +dP/dt	
		34% ↓ PLB	ND	26% ↓ -dP/dt	
		↔ CQS	ND		

(Continued ....)

**Table 1: (continued).**

Model	Species	SR Ca <sup>2+</sup> -cycling proteins		Functional evidences	Ref.
		mRNA	Protein		
PO (8-days, Mild HYP)	Rat	↑ SERCA2a & RyR ↔ CQS	ND	↑ Ca <sup>2+</sup> -uptake	267
(8-days, Severe HYP)		↓SERCA2a & RyR ↔ CQS	ND	↓ Ca <sup>2+</sup> -uptake	
PO (8-weeks, Mild HYP)	Rat	↔ SERCA2a	↔ SERCA2a	25% ↓ Ca <sup>2+</sup> -uptake	30
(8 weeks, Severe HYP)		31% ↓ SERCA2a	20% ↓ SERCA2a	16% ↓ phosphoenzyme 44% ↓ Ca <sup>2+</sup> -uptake 31% ↓ phosphoenzyme	
PO (8-weeks) (16-weeks)	Rat	20% ↓ SERCA2a 35% ↓ SERCA2a	20% ↓ SERCA2a 30% ↓ SERCA2a	65% ↓ Ca <sup>2+</sup> -pump ATPase activity	260
PO (5-days)	Rat (9-months)	↔ SERCA2a ↔ CQS	ND	40% ↑ LVSP	269
	Rat (18-months)	69% ↓ SERCA2a 49% ↓ CQS	ND	23% HYP 30% ↑ LVSP No HYP	

(Continued ....)



**Table 1: (continued).**

Model	Species	SR Ca <sup>2+</sup> -cycling proteins		Functional evidences	Ref.
		mRNA	Protein		
PIHF (1-week)	Dog	↔ SERCA2a & PLB	ND	No change even by development of heart failure	270
PIHF (3-4-weeks)	Dog	ND	28% ↓ SERCA2a 28% ↓ PLB	31% delay in Ca <sup>2+</sup> -removal	271
PIHF (4-weeks)	Dog	48% ↓ SERCA2a 140% ↑ CQS	ND ND	Prolonged Ca <sup>2+</sup> -transient	272
PIHF (8-weeks)	Dog	36% ↓ SERCA2a 23% ↓ RyR ↔ PLB & CQS	52% ↓ SERCA2a ND ND	50% ↓ +dP/dt	262

PO, pressure overload; VO, volume overload; HYP, hypertrophy; PIHF, pacing induced heart failure; SERCA2a, SR Ca<sup>2+</sup>-pump ATPase; PLB, phospholamban; RyR, ryanodine receptor; CQS, calsequestrin; ND, not determined; LVEDP, left ventricular end diastolic pressure; +dP/dt, rate of pressure development; -dP/dt, rate of pressure decay; LVSP, left ventricular systolic pressure; \*, These changes were observed as early as 2 days except for CQS; ↔, No change; ↓, decrease; ↑, increase.

indicates no change in the gene expression in some disease states (**Table 1**). On close observation of **Table 1**, it is very clear that pathologies of different origins have an obvious effect on the SR gene expression as well as SR protein content and SR function. These changes were to varying extents and that can be attributed to several reasons: a) differences in species and models used, b) the severity of the disease may dictate the extent and direction of changes (30, 103), and c) aging seems a critical factor in modulating the expression levels of the genes among SERCA2a levels have been reported to decrease by 28% in aged animals in comparison to young animals (101, 104), and when the age factor is added to a condition of mild hypertrophy it further downregulates the gene (105).

As far as the SR gene-protein-function relationship is concerned, it is difficult to make a conclusive statement since most of the experimental studies were designed to investigate one or two aspects where either the protein or function are missing from the study (29, 106, 107). Nevertheless, what is clear from **Table 1** is that: a) SR genes are regulated in an independent manner (29, 97, 98, 103, 108), b) changes in SR function does not necessarily reflect the status or degree of change in the protein (30), and c) the degree of change in the protein is not essentially a mirror image of the degree of change in the gene expression (96, 97). In general, the data in literature have revealed more gaps in our knowledge and have raised more questions than answers. For example, it needs to be established: a) what are the pre- and post-transcriptional or pre- and post-translational factors contributing to the changes observed under these different

experimental conditions, b) what are the factors contributing to the alterations in the protein function, and c) what is the role of the regulatory mechanisms in inducing the changes in the protein function. These questions indicate that more work needs to be done in this area to reveal the real players in inducing the observed changes in different models of heart disease.

In accordance with the results from the animal model, a decrease in the expression of RyR, SERCA2a and PLB mRNA levels has been observed in human heart failure originating from different etiologies (91, 93, 104, 109-114) (Table 2). The discrepancy in the results was also present in the human studies as no changes in the expression of RyR, SERCA2a, PLB and CQS genes due to dilated cardiomyopathy was reported (92, 113). Nevertheless, human studies revealed very interesting observations. First, it confirmed that a decrease in the gene expression does not indicate a change in the protein levels (91, 93, 104, 113). Likewise, no change in the protein level does not mean that the protein is fully functional (93, 104). Second, the absence of significance in the SR gene expression between cardiac diseases originating from different pathological conditions (93, 114), except for RyR (113), highlights an important issue i.e. the observed alterations are pathologic end results. Such observations raise the need for more studies to reveal the pathogenic factors involved in mediating these changes. These factors can only be detected at the initial stages of the disease at onset of the insult, a condition that is limited only to experimental models. Third,

**Table 2: Alterations in mRNA expression and protein content of the SR Ca<sup>2+</sup>-cycling proteins in human heart failure.**

Model	SR Ca <sup>2+</sup> -cycling proteins		Remarks	Ref.
	mRNA	Protein		
IDCM/ICM	50% ↓ SERCA2a 30% ↓ PLB	↔ SERCA2a ↔ PLB		254
IDCM/ICM	31% ↓ RyR in LV & septum 27% ↓ RyR in RV*	ND ND		275
IDCM/ICM/MCM	30% ↓ RyR in IDCM ↔ RyR in ICM & MCM (with a decreasing trend)	↔ RyR in all models	mRNA normalized to Poly(A <sup>+</sup> ) RNA (No difference when normalized to GAPDH) ↔ RyR binding in all groups	277
IDCM	↔ SERCA2a, PLB & CQS	ND		255
IDCM/IHD/PH	↓ RyR, SERCA2a & PLB ↔ CQS	ND ND	Relative to ANF mRNA	276
IDCM/CAD/HCM/CHD	50% ↓ SERCA2a ↔ CQS	ND		274

(Continued ....)

**Table 2: (continued).**

Model	SR Ca <sup>2+</sup> -cycling proteins		Remarks	Ref.
	mRNA	Protein		
IDCM/CAD/VR	48% ↓ SERCA2a in LV & RV	ND	No disease difference	273
IDCM	54% ↓ SERCA2a 40% ↓ PLB	↔ SERCA2a ↔ PLB	23% ↓ Ca <sup>2+</sup> -ATPase 50% ↓ Ca <sup>2+</sup> -uptake	268
IDCM/ICM	ND	36% ↓ SERCA2a	↓ Ca <sup>2+</sup> -uptake	257
IDCM/CAD	50% ↓ SERCA2a in IDCM 45% ↓ SERCA2a in ICM	↓ SERCA2a		258
IDCM	55% ↓ SERCA2a in IDCM 45% ↓ PLB	↔ SERCA2a ↔ PLB	36% ↓ Ca <sup>2+</sup> -ATPase in IDCM	256
ICM	56% ↓ SERCA2a in ICM 31% ↓ PLB	↔ SERCA2a ↔ PLB	37% ↓ Ca <sup>2+</sup> -ATPase in ICM	

IDCM, idiopathic dilated cardiomyopathy; ICM, ischemic cardiomyopathy; MCM, mixed cardiomyopathy; IHD, ischemic heart disease; PH, pulmonary hypertension; CAD, coronary artery disease; HCM, hypertrophic cardiomyopathy; CHF, congestive heart failure; VR, valvular regurgitation; LV, left ventricle; RV, right ventricle; SERCA2a, SR Ca<sup>2+</sup>-pump ATPase; PLB, phospholamban; RyR, ryanodine receptor; CQS, calsequestrin; ANF, atrial natriuretic factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ND, not determined; \*, not significant; ↔, No change; ↓, decrease; ↑, increase.

heterogeneous expression of SERCA2a mRNA has been reported in a heart failure model due to myocardial infarction (115). In this study a decrease in SERCA2a mRNA was observed in the adjacent non-infarcted area as early as one to three weeks post-operation, whereas no change was observed in the remote-non-infarcted area at three weeks and three months time points although it increased by the first week. The heterogeneity in the expression levels may be one of the possible explanations to the discrepancy in the results reported in literature implicating sampling differences. This study (115) suggested that cardiac failure may be accelerated by the depressed transcript levels in the non-infarcted tissue. In view of the evidence reporting differences in SR regulation in different cardiac layers, it would be interesting and actually essential to investigate this aspect carefully. The significance of this point is vital when considering a gene as a therapeutic target. Fourth, it is important to investigate the changes occurring in the different compartments of the heart, atrium versus ventricle and left versus right ventricle since discrepancies in the expression of different genes is also valid (109, 111)

In summary, since most of the studies in literature were designed to determine the mRNA expression or the protein content of one (116, 117) or two SR proteins and at different stages of cardiac diseases, it may be difficult to draw a conclusive picture of what is the sequence of events. Nevertheless, there are many lessons to be learnt: a) alterations in the levels of mRNA can be attributed to changes at the transcription levels (102), and possibly due to changes in mRNA

stability, b) mRNA expression levels of the SR genes are not co-ordinately regulated, c) depressed SR function may not necessarily be correlated with mRNA expression since depressed  $\text{Ca}^{2+}$ -transport in mild hypertrophy, due to decreased  $\text{Ca}^{2+}$ -pump ATPase activity, was not related to changes in SERCA2a mRNA (30) and d) changes in mRNA levels may not reflect changes in protein content or even protein function since a decrease in SERCA2a and PLB transcripts in human heart failure was accompanied by no change in the protein content of these genes (91, 104, 113).

#### **4. Cellular changes due to ischemia-reperfusion**

##### **a. Ischemia-induced injury**

Myocardial ischemia is defined as the inadequate supply of blood to the myocardium with increased resistance to flow. During ischemia, oxygen deficiency depresses mitochondrial oxidative phosphorylation leading to rapid depletion of high energy phosphate stores (100, 118) and accumulation of  $\text{H}^+$  and inorganic phosphates causing acidosis (119). As an acute adaptive mechanism, the myocardium will switch from aerobic into anaerobic metabolism (glycolysis) which is evident from the marked production of lactic acid (118). When ATP hydrolysis is coupled with lactic acid accumulation it will further decrease the intracellular pH. Cytoplasmic acidification (120) stimulates the  $\text{Na}^+$ - $\text{H}^+$ -exchanger (121, 122) to extrude  $\text{H}^+$  out of the cell leading to a prompt increase in  $[\text{Na}^+]_i$ . Moreover, the depletion of energy (100, 118) will also inhibit the  $\text{Na}^+$ - $\text{K}^+$ -ATPase

activity (119) leading to further increase in  $[\text{Na}^+]_i$ . Subsequently, activation of  $\text{Na}^+$ - $\text{Ca}^{2+}$ -exchanger in the reverse mode which expels  $\text{Na}^+$  out of the cell in exchange for the extracellular  $\text{Ca}^{2+}$  results in the development of intracellular  $\text{Ca}^{2+}$ -overload (123). This sequence of events is consistent with Nuclear Magnetic Resonance studies which have shown an instant rise in  $[\text{Na}^+]_i$  and  $[\text{K}^+]$  in the extracellular space (118) followed by a rise in  $[\text{Ca}^{2+}]_i$  during ischemia (118, 119, 124). The rise in  $[\text{Ca}^{2+}]_i$  was reported to occur during the first 9-15 min of ischemia (100, 124) and was suggested to be an essential cause for the occurrence of contractile dysfunction (100, 124).

Intracellular  $\text{Ca}^{2+}$ -overload is also attributed to other factors such as the accumulation and incorporation of long chain fatty acids into cardiac membranes due to mitochondrial impairment in ischemia. These long chain fatty acids and their derivatives impair the function of membrane proteins, increase the membrane permeability, and thereby contribute to the occurrence of  $\text{Ca}^{2+}$ -overload (125, 126). Furthermore, lack of ATP will decrease the energy dependent pumps responsible for the maintenance of  $\text{Ca}^{2+}$ -homeostasis (127) and thus the depressed SL and SR  $\text{Ca}^{2+}$ -pump ATPase activities may lead to the development of intracellular  $\text{Ca}^{2+}$ -overload (128-130). Thirty minutes of ischemia was shown to depress the SR  $\text{Ca}^{2+}$ -uptake and  $\text{Ca}^{2+}$ -release activities, endogenous SR CaMK phosphorylation of RyR, SERCA2a, and PLB, as well as SR phosphatase activity (129, 130). Longer durations of ischemia decreased both the CaMK as well as the PKA phosphorylation of PLB (131).



It appears that impaired  $\text{Ca}^{2+}$ -cycling due to SR abnormalities may be a leading cause for  $\text{Ca}^{2+}$ -overload in myocardial ischemia. A rise in cytosolic free  $\text{Ca}^{2+}$  from  $0.61 \pm 0.06$  to  $3.0 \pm 0.3 \mu\text{M}$  was accompanied by an increase in the mitochondrial  $\text{Ca}^{2+}$  content (100). This is due to a net  $\text{Ca}^{2+}$ -influx into the mitochondria by the mitochondrial  $\text{Ca}^{2+}$  uniporter.  $\text{Ca}^{2+}$  also binds to a regulatory site on the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$ -exchanger thereby inhibiting the extrusion of  $\text{Ca}^{2+}$  and resulting in mitochondrial  $\text{Ca}^{2+}$ -overload (132). Subsequently,  $3 \mu\text{M}$  of  $[\text{Ca}^{2+}]_i$  was suggested to activate the  $\text{Ca}^{2+}$ -dependent degradative mechanisms leading to cellular membrane damage (100). Moreover, in the first 15-30 min of severe ischemia, there is a rapid activation of the  $\beta$ -adrenergic system leading to ventricular tachycardia and fibrillation (133-135) due to intense release of norepinephrine from the nerve endings in the ischemic zone. Low concentrations of ATP and high levels of cytosolic  $\text{Ca}^{2+}$  leaves the crossbridges permanently attached since they are no longer occupied by ATP forming so called rigor bridges (136). At this point it should be pointed out that during ischemia, the mitochondrial carriers are in a reduced state due to the degradation of the adenine nucleotide pool (137). Thus, the interaction of molecular oxygen trapped within the inner membrane of the mitochondria with the leakage of electrons from the respiratory chain leads to the formation of reactive oxygen species (ROS).

### **b. Reperfusion-induced injury**

Although reperfusion of the ischemic myocardium during early stages is essential to prevent cardiac damage, reperfusion after a certain critical period has been reported to have deleterious effects. These are represented by contractile dysfunction, an increase in infarction size (138), ultrastructural damage and changes in myocardial metabolism which at a later stage lead to cell necrosis (139). This phenomenon “ischemia-reperfusion (IR) injury” is considered to occur during different clinical procedures and was demonstrated by different experimental settings (140) (**Table 3**). At the cellular level, upon reperfusion, the wash out of  $H^+$  will restore myocardial pH and will in turn generate a gradient activating the  $Na^+-H^+$ -exchanger (141-143). Subsequently, there will be an increase in  $[Na^+]_i$  activating the  $Na^+-Ca^{2+}$ -exchanger (143, 144) which in turn causes further increase in  $[Ca^{2+}]_i$ . This effect is also attributed to depressed SL  $Na^+-K^+$ -ATPase activity (128) which is unable to handle the high levels of  $[Na^+]_i$ . Moreover, an increase in  $Ca^{2+}$  entry via the L-type  $Ca^{2+}$ -channel (145) and depressed SL  $Ca^{2+}$ -pump ATPase activity (128) will also participate in the occurrence of  $Ca^{2+}$ -overload. The contribution of intracellular compartments in the genesis of  $Ca^{2+}$ -overload (146) was also reported earlier. This possibility was confirmed by the observed alterations in the SR  $Ca^{2+}$ -uptake, release and ryanodine binding properties observed in the IR hearts (130, 147, 148). These changes were associated with decreased CaMK and PKA phosphorylation as well as the content of the SR  $Ca^{2+}$ -cycling proteins (129, 130).

**Table 3: The experimental and clinical settings of myocardial ischemia-reperfusion.**

Experimental Settings	Clinical Settings
<ul style="list-style-type: none"> <li>❖ In vivo, regional ischemia               <ul style="list-style-type: none"> <li>• Single ischemic episode</li> <li>• Multiple ischemic episodes</li> </ul> </li> <li>❖ In vitro, global ischemia               <ul style="list-style-type: none"> <li>• Isolated heart preparation</li> <li>• Cardioplegic arrest</li> </ul> </li> <li>❖ Exercise-induced ischemia               <ul style="list-style-type: none"> <li>• Cardiac hypertrophy</li> <li>• High-altitude ischemia</li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>❖ Post-thrombolytic therapy</li> <li>❖ Inotropic support after cardiac surgery</li> <li>❖ Angioplasty</li> <li>❖ Coronary bypass graft surgery</li> <li>❖ Cardiac transplantation</li> <li>❖ Angina               <ul style="list-style-type: none"> <li>• Unstable angina</li> <li>• Exercise-induced angina</li> </ul> </li> </ul>

In theory, the restoration of coronary flow and hence oxygen supply to the ischemic muscle is assumed to re-energize the mitochondria to produce ATP for maintenance of the cellular functions. However, the mitochondria utilizes ATP to buffer the high levels of cytoplasmic  $\text{Ca}^{2+}$  rather than replenishing the ATP pool (149) which in turn leads to mitochondrial  $\text{Ca}^{2+}$ -overload. Moreover, considerable evidence has been accumulating showing that reperfusion is accompanied by overflow of norepinephrine from the nerve endings predominantly during ischemia and is then present in the extracellular space (150) and thus participate in the increased uptake of extracellular  $\text{Ca}^{2+}$  into the myocardium (151-153). There is also an ample body of evidence implicating the involvement of ROS in the pathogenesis of cardiac dysfunction in ischemic heart disease. During reperfusion, activated neutrophils and impaired mitochondrial reduction of molecular oxygen lead to electron leakage from the respiratory chain and hence an excessive production of superoxide radical ( $\text{O}_2^{\bullet}$ ) and subsequent formation of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and hydroxyl radical ( $\text{OH}^{\bullet}$ ). These species are intracellular sources for oxidative stress (86, 154), whereas secretion of ROS by phagocytic white blood cells, dysfunctional endothelial cells, or the auto-oxidation of catecholamines may be some of the extracellular sources (90, 137, 155).

##### **5. Mechanisms underlying cardiac dysfunction due to ischemia-reperfusion**

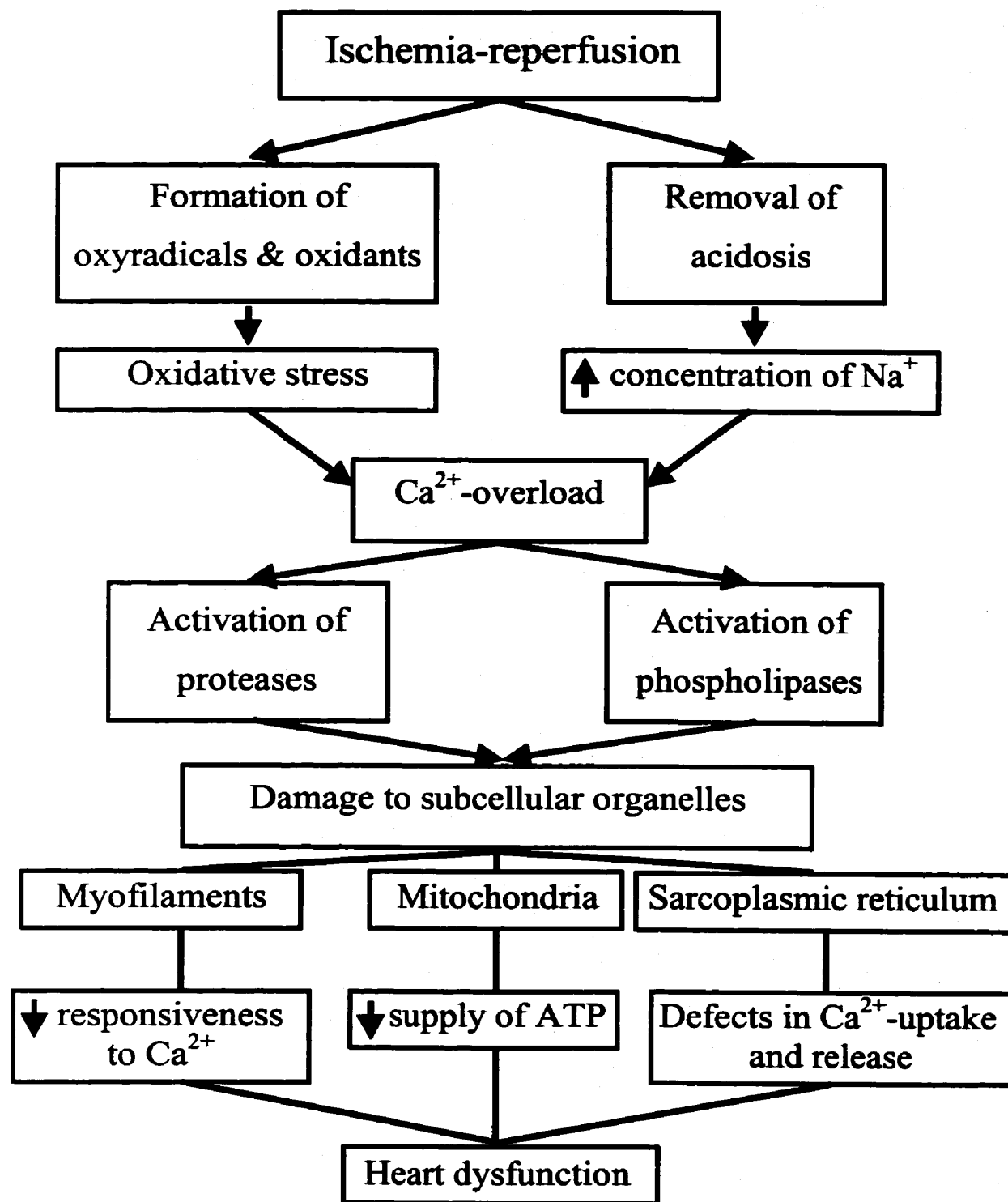
From the above review of literature it is clear that oxidative stress and intracellular  $\text{Ca}^{2+}$ -overload are the two major events occurring in the IR heart

(Figure 3). In this section, we will review the evidences put forward supporting the role of these factors in IR-induced injury.

**a. Oxidative stress**

The deleterious effects of oxidative stress in IR hearts has been under intense investigation due to its importance in cardiac transplantation especially after prolonged periods of ischemia or myocardial preservation (156). The fundamental explanation for the occurrence of oxidative stress is the impairment of the antioxidant defense system, excess generation of ROS or both. The increased levels of ROS under several pathophysiological conditions, that seem to be related to inflammatory processes, are poorly understood. This may be due to difficulties in characterizing their site of origin as ROS are highly labile.

In fact, the role of ROS in IR injury has been shown directly by employing electron paramagnetic resonance spectroscopy (157, 158), trap-phenyl-*N*-tert-butyl nitron (159) and luminal-enhanced ter-butyl-initiated chemiluminescence (160). In IR hearts, oxidative stress was observed to correlate well with cardiac dysfunction (161), a decrease in the antioxidant defense mechanisms (116, 162) and an increase in lipid peroxidation (162, 163) leading to increased membrane permeability. Indirectly, oxidative stress-induced injury was also established by observing the beneficial effects of antioxidants in hearts subjected to IR (127, 129, 164, 165). Since free radicals are very reactive species, they are capable of



**Figure 3:** Schematic representation of the role of oxidative stress and  $\text{Ca}^{2+}$ -overload in IR-induced injury.

attacking virtually any cellular structure. Therefore, the concept was substantiated when hearts or subcellular organelles exposed to oxyradical generating systems produced effects similar to those observed in hearts subjected to IR (166-173). Different ROS such as  $O_2^\bullet$  and  $H_2O_2$  influenced the ionic levels of  $Ca^{2+}$  by depressing the activity of SL  $Ca^{2+}$ -ATPase and  $Na^+$ - $K^+$ -ATPase (170, 174), attenuating the  $\beta$ -adrenergic receptor linked signal transduction system (175, 176) and inhibiting myofibrillar creatine kinase and myofibrillar  $Ca^{2+}$ -ATPase activities (171, 172). Oxidative stress also accounted for the depressed activities of mitochondrial creatine kinase (177),  $Ca^{2+}$ -transport (178, 179) and cytochrome C oxidase (180, 181). Lysosomal hydrolases are released from lysosomes targeted by ROS (135). These cellular changes were correlated with an increase in the lipid peroxidation and were prevented by superoxide dismutase (SOD) and/or catalase (CAT) treatment (167, 171, 172, 175, 176, 182, 183). Moreover, IR hearts treated with SOD plus CAT showed a decrease in infarct size (165), an improvement in cardiac function (127) and SR regulatory function associated with CaMK (129). The depressed SL  $Ca^{2+}$ -pump and  $Na^+$ - $Ca^{2+}$ -exchanger in hypoxic-reoxygenated hearts (184) and  $Na^+$ - $K^+$ -ATPase activities in the IR hearts were also prevented with a combination of SOD plus CAT (185). Using genetically manipulated mice, Chen et al (186) demonstrated a decrease in the IR-induced injury as reflected by improved cardiac performance and decreased lactate dehydrogenase release in transgenic mice overexpressing Mn-SOD. A similar protection was also reported in conscious rabbits genetically treated with adenovirus-mediated SOD transfer

(187).

As one of the targets of oxidative stress, several studies have documented the deleterious effect of ROS on the SR proteins. Hydroxyl radicals caused a decrease in cardiac SR  $\text{Ca}^{2+}$ -pump ATPase activity (188-190) probably by attacking the ATP binding site (190). Exposure of SR vesicles to  $\text{O}_2^\bullet$  and  $\text{H}_2\text{O}_2$  resulted in the progressive loss of RyR binding (191). At the initial stage, the channels are in an open state and this is followed immediately by irreversible dysfunction. On the other hand,  $\text{OH}^\bullet$ ,  $\text{O}_2^\bullet$ , and nitric oxide have all been reported to promote SR  $\text{Ca}^{2+}$ -release by the interaction with sulfhydryl groups of the cardiac and skeletal RyR (189, 190, 192-194). Hydroxyl radical was shown to be released at 60-90 sec and peaks 180-210 sec of reperfusion after 30 min of ischemia especially in fibrillating hearts (135). Hearse (195) suggested that SR dysfunction due to excessive amounts of free radicals generated during reperfusion leading to  $\text{Ca}^{2+}$ -overload is a possible mechanism for arrhythmias. Therefore, due to its wide range of targets which include  $\text{Ca}^{2+}$ -handling mechanisms, oxidative stress may subsequently lead to increased levels of  $[\text{Ca}^{2+}]_i$ .

#### **b. Intracellular $\text{Ca}^{2+}$ -overload**

Fleckenstein (196) was first to describe  $\text{Ca}^{2+}$ -overload as a phenomenon that is intimately involved in cardiac pathology. Since then many studies have been conducted to elucidate the involvement of  $\text{Ca}^{2+}$ -overload in different cardiac diseases. Shen and Jennings (197) reported the role of  $\text{Ca}^{2+}$ -overload in



myocardial IR injury, an observation that has been reinforced by the findings of other investigators (143, 198-201). Elevated levels of intracellular  $\text{Ca}^{2+}$  are known to accompany the development of cardiac contracture (202), cellular damage and cardiac dysfunction (143, 201, 203, 204). It was also shown to be the underlying mechanism for the significant decrease in some membrane phospholipids with an increase in lysophosphatidylcholine content (205). The toxic effect of  $\text{Ca}^{2+}$ -overload includes abnormal metabolism, electrophysiological derangement, disruption of membrane integrity and ultrastructural changes. Moreover, the activation of  $\text{Ca}^{2+}$ -activated proteases (calpains - widely distributed in the myocardium (206, 207) that are involved in cardiac dysfunction) were also observed in the IR heart. These proteases induce their deleterious effect by the hydrolysis of proteins such as troponin-I and troponin-T (208), caldesmon, a cytoskeletal protein (209), as well as SERCA2a and RyR (210, 211). It is also possible that  $\text{Ca}^{2+}$ -activated proteases may attack the endogenous antioxidant proteins such as SOD, CAT and glutathion peroxidase leading to enhanced oxidative stress. The breakdown and release of the myofilament proteins that occur in the IR myocardium have been associated with a decrease in maximum force generation (212).

Likewise, the increase in the levels of  $\text{Ca}^{2+}$  in the mitochondrial matrix results in increased permeability due to either the opening of unselective pores (213, 214) or the accumulation of phospholipids in the mitochondrial membrane due to the activation of phospholipase  $\text{A}_2$  (215, 216). This in turn leads to impaired

oxidative phosphorylation and decreased ability of mitochondria to synthesis ATP (149). Whereas, the energy produced under such a condition is utilized in cycling the excess content of  $\text{Ca}^{2+}$  leading to delayed after-depolarization and arrhythmogenesis (217) and excessive activation of contractile elements to induce cardiac damage (196). From the foregoing discussion it is evident that there is substantial evidence to indicate the deleterious effects of intracellular  $\text{Ca}^{2+}$ -overload and oxidative stress in the myocardium, nevertheless a cause-effect relationship is not yet clear. Thus, due to the interactive nature of oxidative stress and  $\text{Ca}^{2+}$ -overload, it is rather difficult to decide whether these pathogenic factors are causally related or complementary mechanisms of cellular injury (86, 90). To this effect, Opie (218) stated that: "until further experiments are undertaken in the same closed chest model, comparing generators of free radicals with induced calcium overload and free radical scavengers with calcium antagonists or modulators" this issue will not be resolved.

## **6. Changes of SR function by pharmacological interventions**

Pharmacological interventions are considered the most ancient and traditional mode of therapy for heart disease. This strategy significantly contributed to our understanding of the functional importance of proteins and their interaction with other functional proteins or signalling pathways. It is becoming clear that  $\text{Ca}^{2+}$ -related sites would be one of the most important targets for therapeutic interventions to be under investigation.  $\text{Ca}^{2+}$ -related therapy was first started by

the discovery of digitalis which is a  $\text{Na}^+\text{-K}^+\text{-ATPase}$  inhibitor (219). The importance of this drug lies in its ability to indirectly activate  $\text{Na}^+\text{-Ca}^{2+}\text{-exchanger}$  to work in the reverse mode which in turn induces a positive inotropic effect in patients with heart failure. Another approach was to target the SR  $\text{Ca}^{2+}\text{-pump}$  where cardiac SR  $\text{Ca}^{2+}\text{-uptake}$  as well as  $\text{Ca}^{2+}\text{-stimulated ATPase}$  activity were normalized when the diabetic animals were treated with verapamil, an L-type  $\text{Ca}^{2+}\text{-channel}$  blocker (220). An improvement in SR  $\text{Ca}^{2+}\text{-uptake}$  was also observed when IR hearts were treated with diltiazem,  $\text{Ca}^{2+}\text{-antagonist}$  (221). This protection was accompanied with improved cardiac performance as well as maintained high levels of energy.

Although the efficiency of antioxidant treatment is controversial, there is an increasing body of evidence to suggest the protective effects of antioxidants and oxyradical scavengers at various levels. Earlier studies have shown that the treatment of the heart with a combination of SOD plus CAT improved myocardial functional recovery after IR (158, 222). The combination of SOD plus CAT was observed to protect oxyradical-induced changes in membrane lipids, partially attenuate the ionic imbalance, and significantly reduce ventricular fibrillation in the IR hearts (134, 135). IR hearts treated with a combination of SOD plus CAT showed improved SR  $\text{Ca}^{2+}\text{-uptake}$ ,  $\text{Ca}^{2+}\text{-release}$  and RyR binding (129). This protection was accompanied by enhanced phosphorylation of SR  $\text{Ca}^{2+}\text{-cycling}$  proteins. SOD plus CAT also reversed the inhibition of SR  $\text{Ca}^{2+}\text{-uptake}$  and  $\text{Ca}^{2+}\text{-ATPase}$  activity in SR vesicles treated with activated leukocytes (223).

The renin-angiotensin system has been implicated in inducing oxidative stress in vascular and endothelial smooth muscle cells and may be an important mechanism underlying the pathophysiology of hypertension (224). This role was evident when increased angiotensin-converting enzyme (ACE) activity and reduced endothelium-dependent vasodilation were observed in the aortae of hypertensive rats (225). Hypertension induced by angiotensin (Ang-II) infusion has been reported to be mediated by elevated vascular  $O_2^\bullet$  production (226, 227) and increased activity of NADH/NADPH oxidase (228). The significance of ACE inhibition stems from the ability of ACE inhibitors (ACE-I) to prevent the conversion of Ang-I to Ang-II (229) and thereby reduce Ang-II induced oxidative stress. IR hearts as well as animals with chronic heart failure exhibited depressed cardiac performance and SR  $Ca^{2+}$ -uptake,  $Ca^{2+}$ -release and  $Ca^{2+}$ -ATPase activities (230-233). These effects were prevented when hearts were treated with captopril (230-232), tradolapril (233), or losartan (Ang-II receptor blocker  $-AT_1$ ) (230, 232). This protection was accompanied with improved levels of SERCA2a, PLB and RyR protein content.

Increased activity of the sympathetic system (234) as well as 100-1000 fold increase in the release of norepinephrine from the nerve endings within 20-40 min of ischemia (235) has also been reported to mediate myocardial cell damage. It should be pointed out that  $\beta$ -adrenoceptor ( $\beta$ -AR) blockers have been shown to provide protection against some of the abnormalities, including a reduction of the infarct size (236), an attenuation of arrhythmias (133), an improvement in the

ventricular function and an overall decrease in mortality (237) which are commonly observed in ischemic heart disease. Although the exact mechanisms for the cardioprotective action of these agents on the ischemic heart are not yet fully understood,  $\beta$ -AR blockers are known to lower myocardial oxygen consumption as a consequence of reduced contractility and heart rate, increase oxygen delivery due to coronary artery dilation (238, 239), as well as due to their membrane stabilizing (240) and antioxidant (241, 242) properties. These findings have formed the basis for developing therapeutic strategies in which  $\beta$ -AR blockers are considered to be beneficial for the treatment of IR-induced injury. Rat hearts exposed to myocardial infarction, model of ischemic cardiomyopathy, showed an improvement in cardiac performance, SR  $\text{Ca}^{2+}$ -uptake,  $\text{Ca}^{2+}$ -release and  $\text{Ca}^{2+}$ -ATPase activities when treated with propranolol (243). This protection was accompanied by recovery of SERCA2a protein content. Similar effects were observed when animals were treated with verapamil ( $\text{Ca}^{2+}$ -channel blocker). D-sotalol and tedisamil were found to prevent  $\text{Ca}^{2+}$ -overload in cardiomyocytes by a cAMP-dependent SR  $\text{Ca}^{2+}$ -uptake mechanism (244). Sotalol, on the other hand enhanced the SR function indirectly by reducing  $\text{Ca}^{2+}$ -extrusion via the  $\text{Na}^+$ - $\text{Ca}^{2+}$ -exchanger (245). This mechanism will allow more SR loading with  $\text{Ca}^{2+}$ , which will be released by the next wave of depolarization.

## **7. Changes in SR function due to ischemic-preconditioning**

Ischemic preconditioning (IP) was first described by Reimer et al (246) when repetitive cycles of IR protected the heart against a prolonged ischemic insult. Studies in this area suggested that this protection appears in two phases: phase I (classical preconditioning) which occurs 1-3 hours after the onset of the first ischemic insult (247) and phase II (second window of protection) which appears 12-72 hrs later (248). Several mediators have been associated with the cardiac protection observed among which are: norepinephrine, Ang-II, adenosine, nitric oxide and activation of mitogen activated protein kinase and protein kinase C (249). IP has been frequently shown to improve left ventricular contractile function (130, 250-252), reduce arrhythmias (253, 254) and infarct size (247). Several studies have shown that the improvement in left ventricular developed pressure, rate of relaxation and contraction in the IR and  $\text{Ca}^{2+}$ -depleted/repleted hearts were related to improved SR  $\text{Ca}^{2+}$ -uptake,  $\text{Ca}^{2+}$ -release and ryanodine binding properties (130, 251, 255). The duration (255) and number (256) of the ischemic cycles were considered as important factors determining the level of protection. Nevertheless this protection was negatively related to age due to increased susceptibility to ischemia by aging (257). The attenuation in the SR protein content and regulatory mechanisms was also prevented by IP (130, 251). These studies have shown that adenosine receptors, protein kinase C and CaMK may be possible mediators in the protection observed (130, 251). The beneficial effect of IP resides in its ability to attenuate the rise in  $[\text{Ca}^{2+}]_i$ , improved cardiac

energetics and reduced stimulation of  $\text{Na}^+\text{-H}^+$ -exchanger and  $\text{Na}^+\text{-Ca}^{2+}$ -exchanger during ischemia (252). In general, the key factor in the induced protection lies in minimizing the ionic imbalance.

In view of the literature reviewed above, it is clear that  $\text{Ca}^{2+}$ -overload, oxidative stress and excessive release of catecholamines are major factors contributing to IR-induced injury (86, 249). Nevertheless, these mediators have been also shown to be some of the underlying mechanisms involved in the IP-induced protection at the level of the SR. When  $[\text{Ca}^{2+}]_i$  loading was induced in a pre-ischemic phase whether by activating the release or blocking  $\text{Ca}^{2+}$ -uptake there was an improvement in cardiac performance (258), indicating that  $\text{Ca}^{2+}$  released from the SR may participate in the cardioprotection against IR-induced injury. The functional recovery induced by IP was masked when the SR membranes were treated with reducing agents (259). This treatment prevented the improvement in SR  $\text{Ca}^{2+}$ -release and ryanodine binding properties observed with IP due to the oxidation of sulfhydryl groups of RyR. Cardiac trabeculae preconditioned with norepinephrine showed better functional recovery when compared with metabolically inhibited controls (260); an improvement that persisted even with the inhibition of the SR  $\text{Ca}^{2+}$ -release. This study suggested that IP protected the  $\text{Ca}^{2+}$ -stimulated ATPase activity. Therefore, from a therapeutic point of view the discovery of the mediators and the signalling pathways involved in the protective effects of IP can be used for the generation of agents that mimic these effects.

## **8. Gene therapy and pharmacological approaches for the treatment of SR dysfunction in heart failure**

Although patients with heart failure are characterized by multiple cardiac abnormalities, it is clear that alterations in  $\text{Ca}^{2+}$ -handling are the primary cause of cardiac dysfunction (85, 88). Gene therapy has been instrumental in enhancing our understanding of different proteins since each protein can be studied in isolation by specific genetic manipulation of their expression, and therefore emphasizing the role of these proteins in modulating cardiac performance. Studies targeting SERCA2a for gene therapy were tackled by two different approaches: transgenic models and adenovirus mediated overexpression. Transgenic animals were used to enhance the expression of SERCA2a (261, 262) or replace it with a functional isoform such as SERCA1a (263, 264). Although these studies have shown an enhanced levels of  $\text{Ca}^{2+}$ -uptake and increased rate of relaxation and amplitude of  $\text{Ca}^{2+}$ -transient, the genetic manipulation was accompanied with reduced endogenous SERCA2a expression in the case of SERCA1a overexpression (263, 264). Replication-deficient recombinant adenovirus gene transfer has been shown to be a more efficient technique since it does not interfere with the expression of other endogenous genes (265-267). Overexpression of SERCA2a in isolated myocytes enhanced cardiac contraction and relaxation (261). The correlation between enhanced expression of SERCA2a mRNA, its protein content and the dynamic intracellular  $\text{Ca}^{2+}$ -regulation has been observed when adenovirus-mediated expression of SERCA2a in ventricular neonatal cardiomyocytes



shortened  $\text{Ca}^{2+}$ -transients and enhanced  $\text{Ca}^{2+}$ -uptake (265-267). Although the increase in activity was disproportionate to the increase in protein content, improved relaxation was attributed to increased SERCA2a to PLB ratio, with no effect on the expression of PLB. Moreover, this technique was also successful in expressing a functional pump in cardiomyocytes from failing human hearts (268). The general observation of all studies carried out on the genetic manipulation of the SERCA gene is that SERCA2a plays a rate limiting role during cardiac relaxation and therefore it is critical for the occurrence of cardiac contractility (263, 266).

It has been argued that PLB antisense expression may have an advantage over the SERCA2a overexpression (269) since the decrease in PLB levels is less than that observed for the SERCA2a in the failing heart (23, 270) and the decreased SERCA2a sensitivity for  $\text{Ca}^{2+}$  is reduced due to depressed levels of PLB phosphorylation (271). The genetic manipulation of this protein was also an important approach since enhanced cardiac contractility was reported in transgenic model with PLB knock out (73), while PLB overexpression attenuated  $\text{Ca}^{2+}$  kinetics (272). PLB knock out mice were able to retain SERCA2a phosphorylation by CaMK and have shown higher levels of SR  $\text{Ca}^{2+}$ -uptake and SR  $\text{Ca}^{2+}$ -loading (273, 274). Moreover, the abnormalities in  $\text{Ca}^{2+}$ -handling were also addressed by weakening the interaction between SERCA2a and PLB by the expression of mutant SERCA2a or PLB proteins (261). This in turn increased  $\text{Ca}^{2+}$ -uptake due to the ablation of the inhibitory effect of PLB on the pump. While overexpression of

SERCA2a was accompanied with enhanced contractile properties of the cells (266), overexpression of PLB showed opposite results that were reversed by co-overexpression of SERCA2a (265). In an attempt to investigate the role of PLB-SERCA2a interaction in the modulation of the pump activity, cloning of a PLB mutant at two amino acids within the PLB molecule into adenovirus to infect adult rat and rabbit myocytes showed an acceleration of SERCA2a activity (275).

Other approaches have been adopted to test the possible compensatory mechanisms for improving  $\text{Ca}^{2+}$  transport. Although it is well known that SL  $\text{Ca}^{2+}$ -pump ATPase plays a less significant role in regulating  $\text{Ca}^{2+}$  movement during cardiac relaxation and it has different  $\text{Ca}^{2+}$  to ATP stoichiometry (one  $\text{Ca}^{2+}$  ion transported/one molecule of ATP) (276) as compared to the SR SERCA2a (two  $\text{Ca}^{2+}$  ions transported/one molecule of ATP), it was hypothesised that overexpression of this protein may improve cardiac relaxation (277). In this study (277) transgenic animals exhibited an increase in the expression of the pump but there was no difference in the hemodynamic parameters,  $\text{Ca}^{2+}$  transients or L-type  $\text{Ca}^{2+}$  current as compared to the controls. However, the authors reported an interesting observation that is neonatal cardiomyocytes overexpressing the SL  $\text{Ca}^{2+}$ -pump shows an increase in the rate of cardiac protein synthesis, suggesting a significant role of the pump in the regulation of cellular growth.

Although molecular biology techniques are opening new horizons for us to tackle the heart failure problems, serious precautions have to be taken into consideration. First, genes have to be overexpressed in a controlled fashion which

may not interfere with the machinery that transcribe and transport the mRNA to reach its destination to be translated. Second, it is important to assure that the overexpressed protein is not only translated but also incorporated into the membrane and is functional (261). Third, the overexpression of a protein should not interfere with the expression of other proteins and can be accommodated with respect to the regulatory mechanisms that function under normal conditions. Fourth, a protein overexpression as in the case of SERCA2a should not create a dilemma as far as the energy turnover is concerned (278). Fifth, in view of the differences in  $\text{Ca}^{2+}$ -handling (115) and the heterogeneity of gene expression in different areas of the heart tissue (274), to handle the transmural gradient across the myocardium, it is important to consider these aspects while overexpressing a specific gene. Finally, more attention has to be paid to the possibility of applying these approaches to the main target that is the human heart failure.

In parallel to the genetic manipulation employed to improve cardiac function in diseased conditions, other studies adopted several pharmacological manoeuvres among which was the transcriptional regulation of a gene. There are several lines of evidence showing that thyroid hormone is an important transcriptional regulator (enhancer) that modulates the expression of SERCA2a gene. First, thyroid hormone treatment of the hypertrophied heart enhanced the expression of SERCA2a mRNA with concomitant decrease in PLB mRNA (29). Whereas, hypothyroidism caused a decrease in SERCA2a transcript with no changes in PLB levels of expression (29). Second, three different thyroid hormone-response

elements were delineated in the promoter of SERCA2a gene (279). Third, neonatal cardiomyocytes treated with thyroxin have shown an increase in the expression of SERCA2a at the level of both mRNA and protein (44, 280, 281). This increase was accompanied by enhanced  $\text{Ca}^{2+}$ -uptake which was independent of PLB phosphorylation (44). Although, the upregulation of the SERCA2a gene was accompanied with improved SR  $\text{Ca}^{2+}$ -handling, thyroxin is not an ideal pharmacological agent for several reasons among which is the tachycardia-induced effect of the doses required to enhance cardiac performance (282, 283). Moreover, thyroid treatment enhances the expression of PLB (284) which in turn may affect the ratio of SERCA2a to PLB then nullify the effect of enhanced SERCA2a expression. From a functional point of view a modified analogue of thyroid hormone (3,5, diiodothyropropionic acid-DITPA) was considered more favourable for the treatment of failing heart than thyroxin (282, 283).

Etomoxir, a compound that shifts the cardiac metabolism from fatty acid to glucose oxidation, has been also suggested as another transcriptional modulator (285). Etomoxir treatment of pressure overloaded hearts prevented the depression in SR gene expression (286). Although this beneficial effect did not reduce the extent of cardiac hypertrophy, the treatment prevented the transition to the heart failure stage. Moreover, the improvement in SR  $\text{Ca}^{2+}$ -ATPase activity was correlated with reduced levels of serum lipids in pressure overloaded (287) and chronic diabetic hearts (288).

In view of their therapeutic significance at the levels of cardiac performance and SR function in a model of myocardial infarction (230, 231, 233), inhibitors of the renin-angiotensin system were also tested for their role in the transcriptional regulation of the SR genes. Different ACE-Is such as captopril,trandolapril, and perindopril, and an AT<sub>1</sub> blocker (losartan) showed protection at the level of SR gene expression in pressure overload induced heart failure and cardiomyopathy (230, 231, 233, 289). Similar recovery was obtained when transgenic animals expressing renin2d gene, to induce hypertensive cardiomyopathy, were treated with captopril or Bay 10-6734, an AT<sub>1</sub> blocker (290). Nevertheless, both captopril and losartan failed to protect the SR gene expression in IR model although cardiac performance and SR functions showed significant recovery (232). It was suggested that the protection rendered by the ACE-I containing SH groups may be attributed to their antioxidant effect (291). Moreover, the improvement in cardiac performance was related to improved SR function. The upregulation in the expression of SERCA2a, RyR and PLB, possibly regulated by the activation of PKC (292), was considered a contributing factor in this recovery (233, 292, 293).

TCV-116, an Ang-II antagonist, showed a differential protection at the level of gene expression in volume overload (98). As compared to sham-operated rats, volume overload caused significant decrease in SERCA2a, RyR, and PLB mRNA with no changes in the expression of CQS. The treatment recovered the levels of SERCA2a and PLB but not RyR. Nevertheless, in view of the role of pressure overload in modulating gene expression (99, 102), Hashida et al (98) suggested

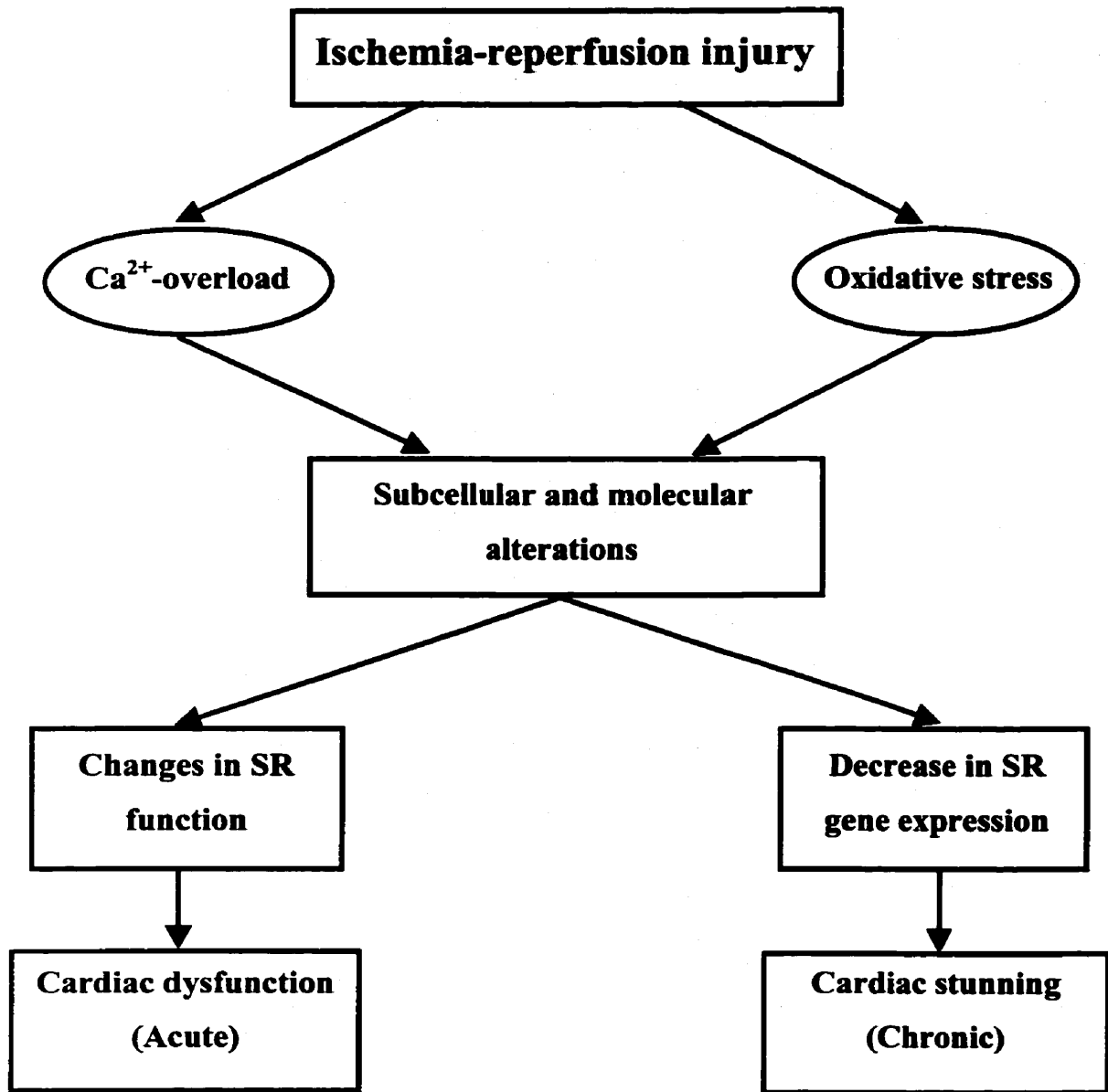
that the antihypertensive effect of TCV-116 may have played a role in the protection rendered on the expression levels of the genes.

Several other pharmacological interventions have been also put into trial. Recently, it was reported that treatment with endothelin receptor blocker improves the expression of the SR genes in heart failure and that was reflected in the increased survival rate and functional recovery (294).  $\beta$ -blockers and  $\text{Ca}^{2+}$ -antagonists induced their protective effect on cardiac performance by protecting SERCA2a protein and gene expression (243). In this area of therapy, drugs that can target the interaction between SERCA2a and PLB may be of significance since they can weaken the protein-protein interaction leading to relieved inhibition from SERCA2a and thus increase SR activity. This interaction can also be manipulated by the activation of CaMK or the enhancement of cAMP levels, nevertheless some caution should be exercised since these signaling pathways can target other proteins. Protein phosphatase inhibitors may have potential therapeutic value since protein phosphatase activity was elevated in heart failure suggesting enhanced dephosphorylation (295).

## II. STATEMENT OF THE PROBLEM AND HYPOTHESIS TO BE TESTED

Cardiac dysfunction is known to occur immediately upon reducing the blood flow to the heart and persists even after the restoration of coronary perfusion (myocardial stunning). These acute and chronic abnormalities in cardiac performance have been partially attributed to depressed SR function in the ischemic and IR hearts. Several studies have reported that defects in the protein content and gene expression of the SR  $\text{Ca}^{2+}$ -cycling proteins may participate in cardiac dysfunction in different types of heart failure and cardiomyopathies; however, very little information regarding such changes in IR hearts is available in the literature. Accordingly, this study was undertaken to test the hypotheses that IR may induce alterations in SR function during two stages of the ischemic heart disease: a) the acute phase of cardiac dysfunction which may be associated with a decrease in SR  $\text{Ca}^{2+}$ -transport activities and protein content, and b) the chronic phase represented by the persistent cardiac dysfunction even after restoration of the coronary flow may be a consequence of pre-existing changes in SR gene expression (**Figure 4**).

Although myocardial ischemia results in contractile dysfunction, the exact pathogenic mechanisms underlying changes upon reperfusion of the ischemic heart are not fully understood. Several factors including intracellular acidosis, depression in the level of ATP, accumulation of metabolites, intracellular  $\text{Ca}^{2+}$ -overload, formation of ROS, glucose-lack and hypoxia have been implicated as

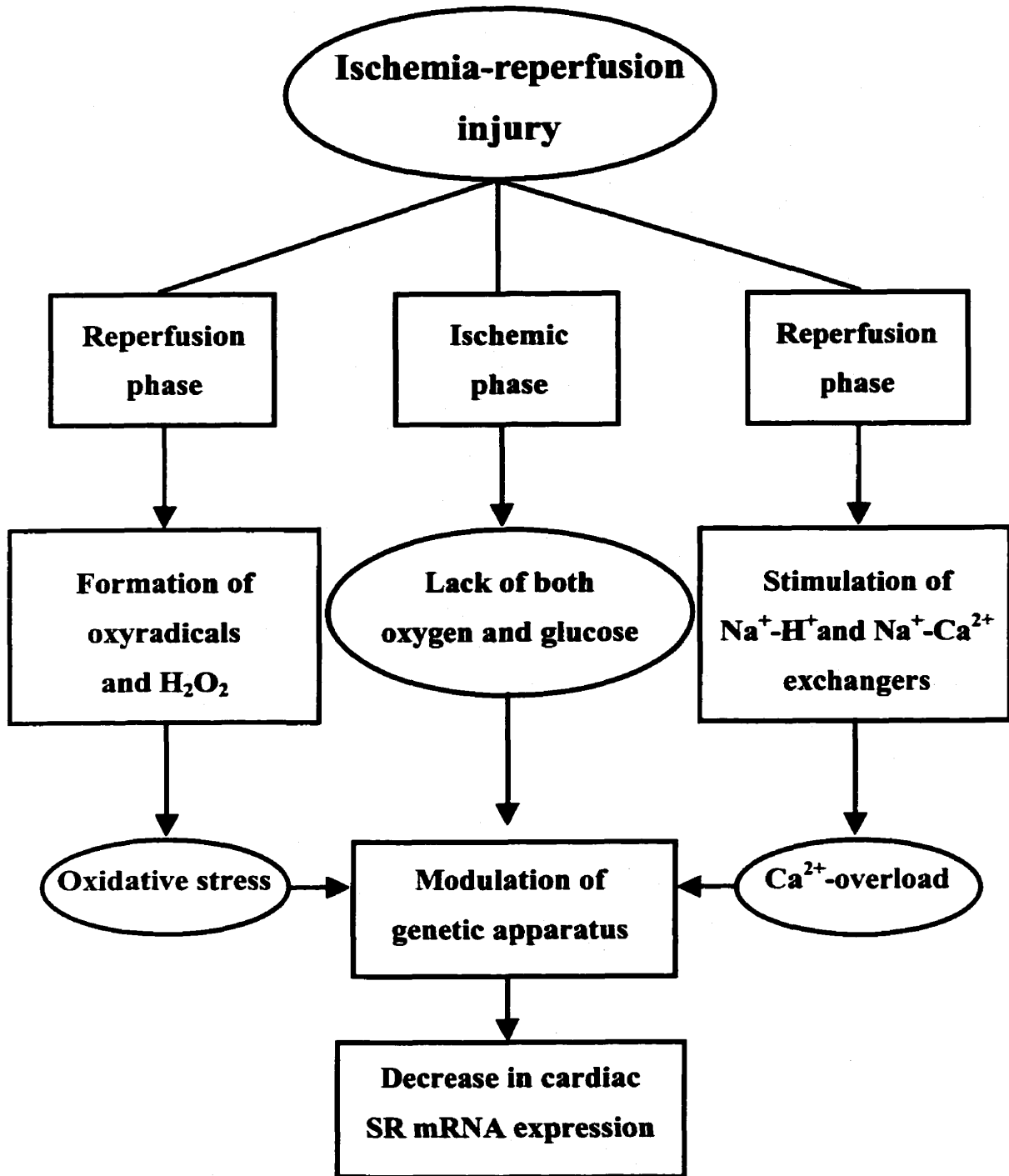


**Figure 4:** Schematic representation of the hypothesized mechanisms involved in the acute and chronic cardiac and SR dysfunction due to ischemia-reperfusion injury.



mediators of IR-induced injury in the heart. However, it is not clear whether the IR-induced changes occur due to the ischemic phase or the reperfusion phase. This study addressed the hypothesis that oxygen-lack and glucose-lack (in a synergistic manner) that occur during the ischemic phase and the oxidative stress and intracellular  $\text{Ca}^{2+}$ -overload, that occur during the reperfusion phase, may contribute to cardiac dysfunction observed in the IR hearts (Figure 5).

In view of the occurrence of oxidative stress and increased levels of catecholamines during reperfusion, we tested the therapeutic potential of antioxidants (SOD plus CAT) and  $\beta$ -adrenergic blockers (atenolol or propranolol) in preventing the IR-induced changes in SR function in the heart. Since ischemic preconditioning is known to produce beneficial effects against the IR-induced and  $\text{Ca}^{2+}$ -depletion/repletion-induced changes in cardiac performance, the effects of this intervention on IR-induced as well as  $\text{Ca}^{2+}$ -depletion/repletion-induced depression in cardiac SR mRNA expression were also investigated.



**Figure 5:** Schematic representation of the hypothesized mechanisms involved in the modulation of SR mRNA expression due to ischemia-reperfusion injury.

### **III. 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 concerning the Care and Use of Experimental Animal (Volume 1, 2<sup>nd</sup> Edition, 1993).

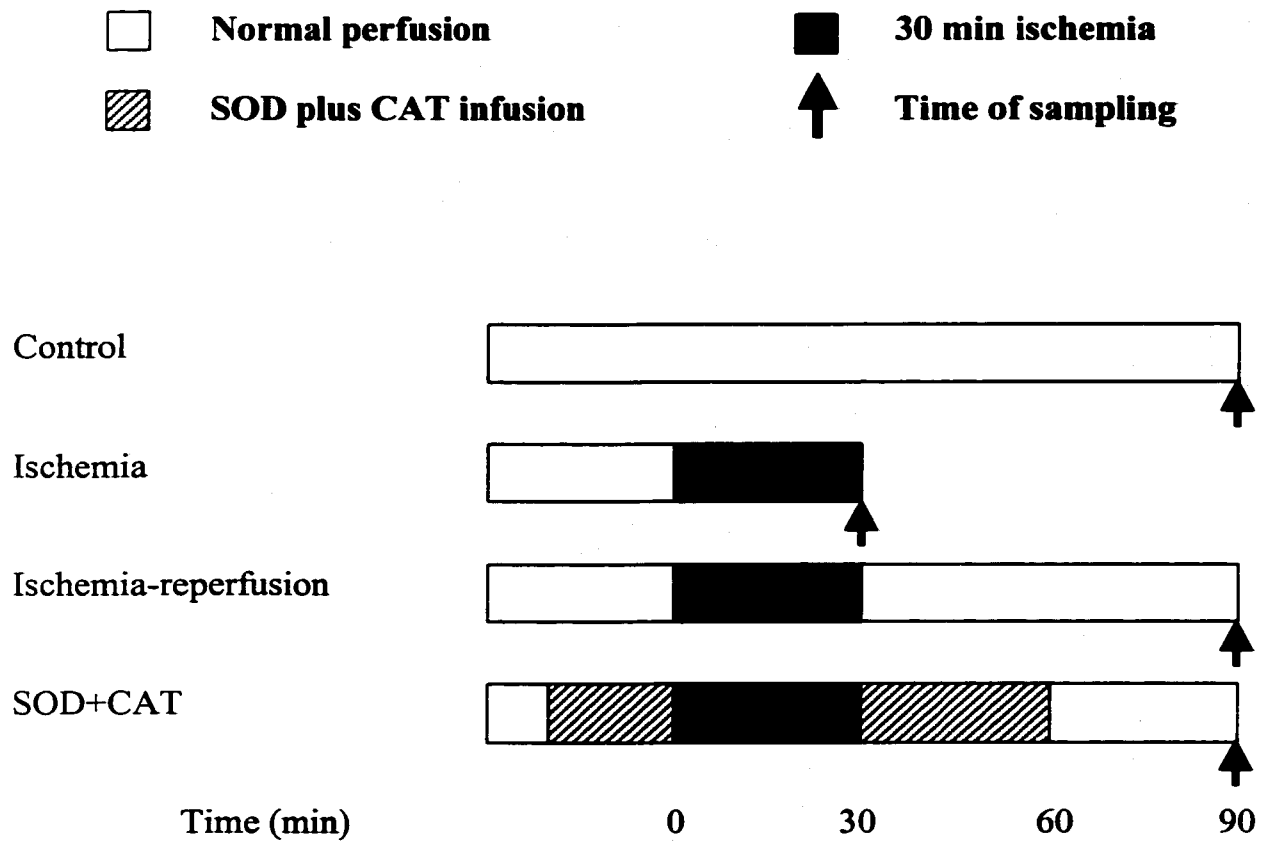
#### **1. Perfusion of isolated rat hearts**

Male Sprague-Dawley rats (300-350 g) were anaesthetised with a mixture of ketamine (60 mg/Kg) and xylazine (10 mg/Kg) that was administered intraperitoneally. Hearts were rapidly excised, cannulated to the Langendorff apparatus and perfused at a constant flow of 10 ml/min using Krebs-Henseleit medium (K-H) gassed with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>, pH 7.4 (147). The composition of K-H buffer was (in mM): 120 NaCl, 25 NaHCO<sub>3</sub>, 4.7 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, 1.25 CaCl<sub>2</sub> and 11 glucose. The hearts were electrically stimulated (Phipps and Bird stimulator) at a rate of 300 beats/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) for the left ventricular systolic pressure and left ventricular end diastolic pressure (LVEDP) measurements; the left ventricular developed pressure (LVDP) was the difference between left ventricular systolic and diastolic pressures. At the beginning of the experiment, LVEDP was adjusted to 10 mm Hg and the left ventricular pressures were differentiated to estimate the rate of ventricular pressure development

(+dP/dt) and the rate of ventricular pressure decline (-dP/dt) using the Acknowledge 3.5.3. software for Windows (BIOPAC SYSTEM INC., Goleta, CA). Data were recorded online through an analogue-digital interface (MP 100, BIOPAC SYSTEM INC), stored and processed using the Acknowledge 3.5.3. software for Windows. All hearts were stabilized for a period of 30 min with K-H medium and maintained at a constant temperature (37°C) throughout the experiments. The hearts were then randomly distributed among different experimental groups.

## 2. Protocol for ischemia-reperfusion

After a 30 min period of stabilization, hearts were divided into four groups (**Figure 6**): (a) control hearts (C): perfused for 30, 60 and 90 min with normal K-H medium and since no differences were observed between different controls in all studied parameters the values were pooled, (b) global ischemia (I): induced by stopping the coronary flow for 30 min, (c) ischemic-reperfused hearts (IR): exposed to 30 min of global ischemia followed by 60 min of reperfusion, (d) IR-treated hearts: IR hearts treated with a mixture of superoxide dismutase (SOD: 3,500 U/mg from Bovine erythrocytes) plus catalase (CAT: 25,000 U/mg from Bovine liver). The mixture was infused into the perfusion medium via a side arm close to the cannula for 20 min before inducing ischemia as well as for 30 min during the reperfusion period. The final concentrations of SOD ( $5 \times 10^4$  U/L) and CAT ( $7.5 \times 10^4$  U/L) in the perfusion medium were according to previous reports

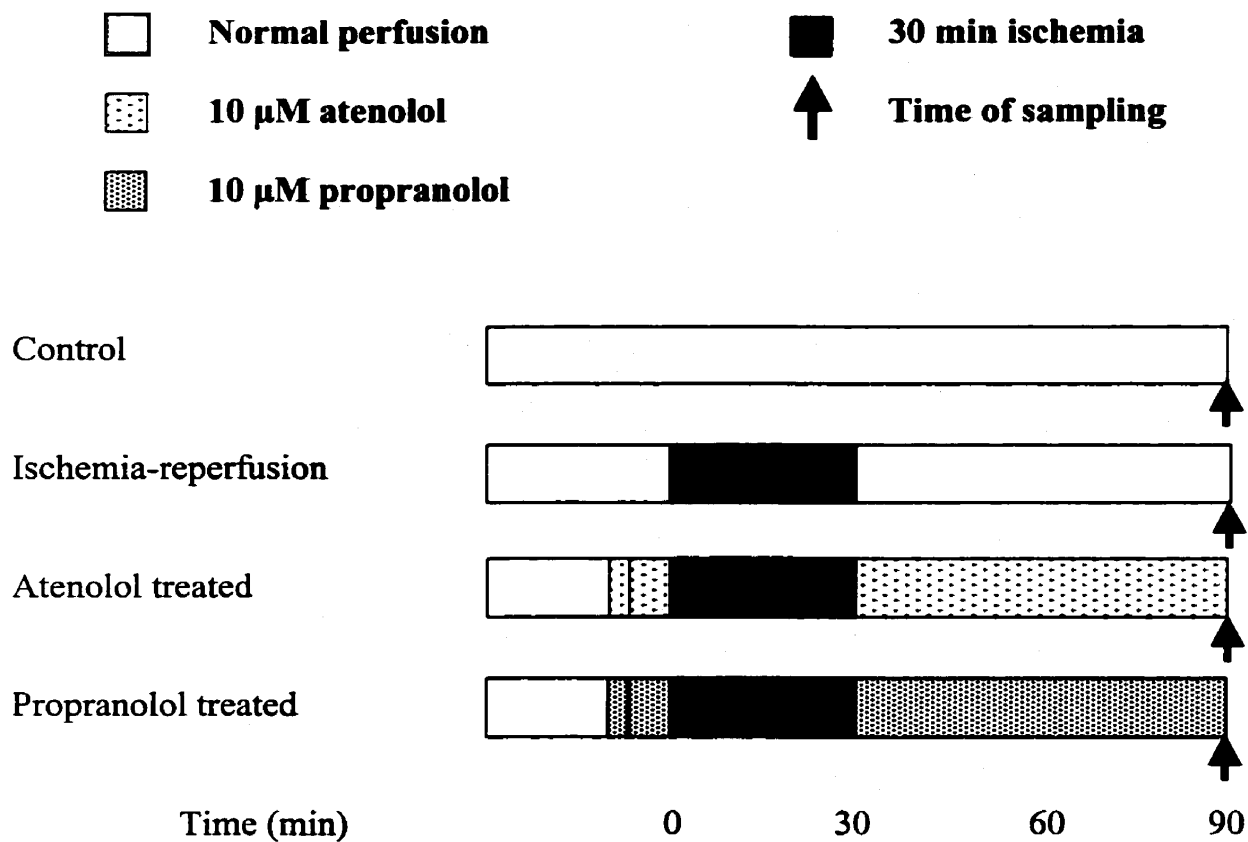


**Figure 6:** Schematic representation of the experimental design for ischemia-reperfusion with and without SOD plus CAT.

(127, 184). Preliminary experiments revealed that when used alone SOD or CAT had no beneficial effects on IR-induced changes reported in this study.

### 3. Protocol for $\beta$ -adrenoceptor blockade in IR hearts

After a 30 min period of stabilization, hearts were divided into four groups (**Figure 7**): (a) control hearts (C): perfused for 90 min with normal K-H medium, (b) ischemic-reperfused hearts (IR): exposed to 30 min of global ischemia followed by 60 min of reperfusion, (c) atenolol treated hearts: IR hearts treated with 10  $\mu$ M atenolol, a  $\beta_1$ -specific blocker, (d) propranolol treated hearts: IR hearts treated with 10  $\mu$ M propranolol, a non-specific  $\beta$ -blocker. Atenolol and propranolol were infused just above the perfusion cannula for 10 min before inducing ischemia as well as for 60 min during reperfusion (296). Atenolol and propranolol were purchased from the Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). The selection of 10  $\mu$ M concentration of both atenolol and propranolol for use in this study was based on the work of other investigators (297, 298). Furthermore, both atenolol and propranolol at 10  $\mu$ M concentrations were observed to prevent the positive inotropic effect of 1  $\mu$ M isoproterenol ( $225 \pm 7.6\%$  increase in the LVDP; N = 4) by 98 and 97%, respectively, under the experimental conditions employed in this study.



**Figure 7:** Schematic representation of the experimental design for ischemic-reperfused hearts treated with atenolol or propranolol.

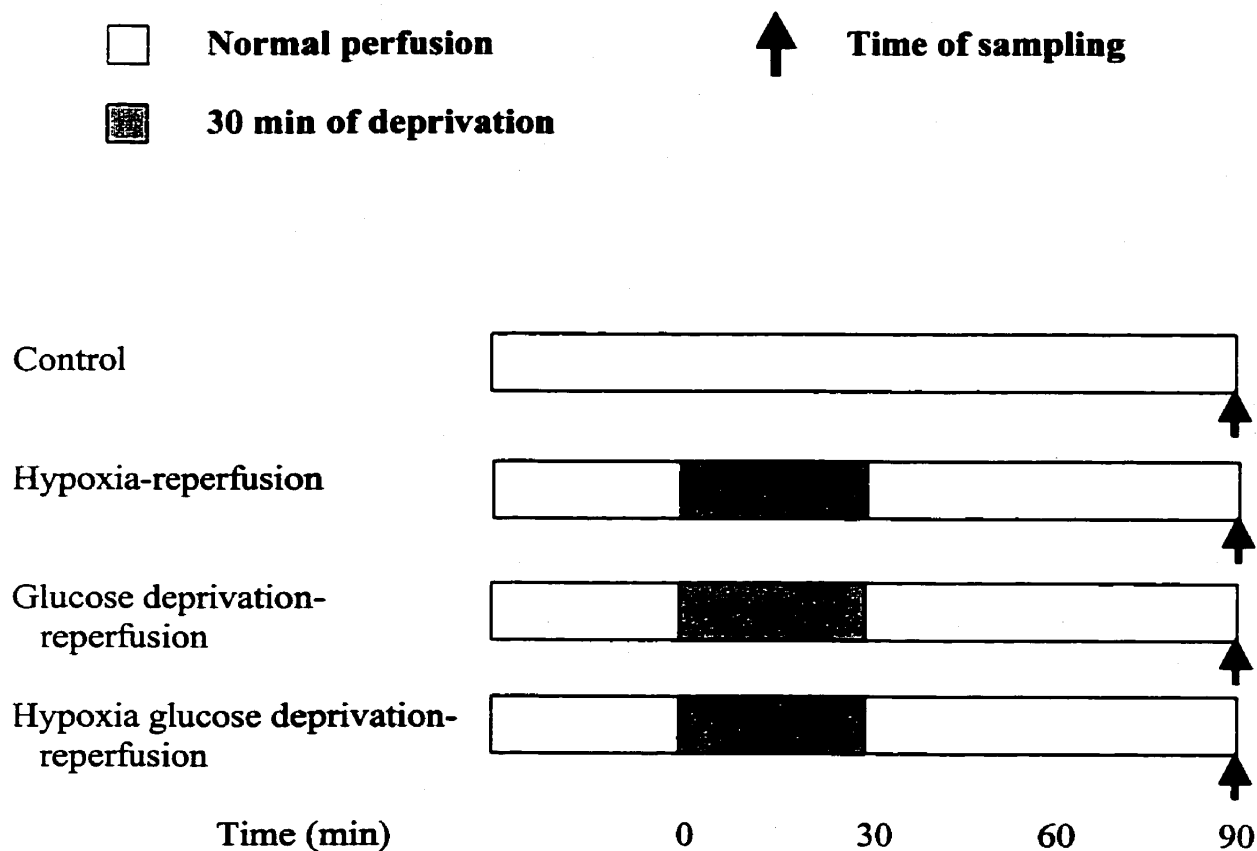
#### 4. Protocol for hypoxia and/or glucose deprivation-reperfusion

After a 30 min period of stabilization, hearts were divided into four groups (**Figure 8**): (a) control hearts (C): perfused for 90 min with normal K-H medium, (b) hypoxic-reperfused hearts (HR): perfused for 30 min with K-H medium gassed with 95% N<sub>2</sub> and 5% CO<sub>2</sub>, pH 7.4 followed by 60 min of perfusion with normal K-H medium, (c) glucose deprived-reperfused hearts (GR): perfused for 30 min with glucose-free K-H medium gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> where glucose was replaced with Tris-HCl to maintain osmolarity, pH 7.4 followed by 60 min of perfusion with normal K-H medium, (d) hypoxic glucose deprived-reperfused hearts (HGR): perfused for 30 min with glucose-free K-H medium gassed with 95% N<sub>2</sub> and 5% CO<sub>2</sub>, pH 7.4 followed by 60 min of perfusion with normal K-H medium. The perfusion procedures were adapted according to previous studies (299-301).

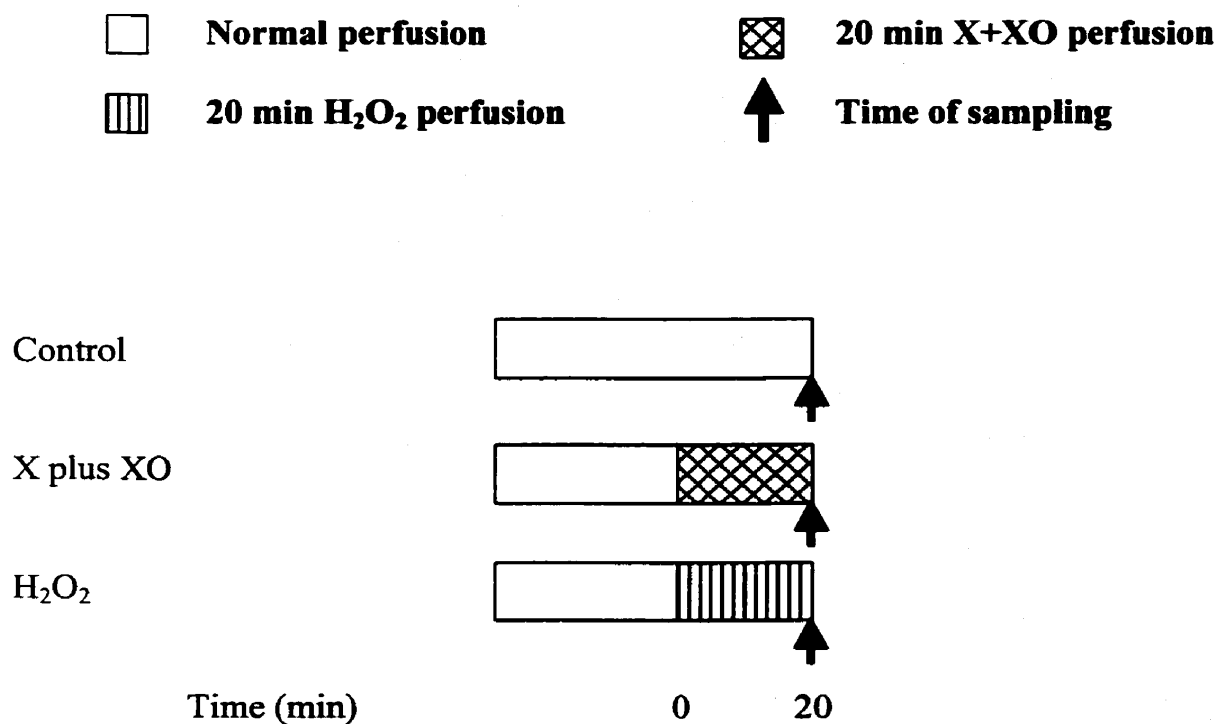
#### 5. Protocol for perfusion with reactive oxygen species

After a 30 min period of stabilization, hearts were divided into three groups (**Figure 9**): (a) control hearts (C): perfused for 20 min with normal K-H medium, (b) xanthine plus xanthine oxidase hearts (X+XO): treated for 20 min with a mixture of xanthine (X: 2 mM) and xanthine oxidase (XO: 0.03 U/ml) dissolved in K-H medium and mixed for 60 min to ensure sufficient generation of the superoxide radical, (c) H<sub>2</sub>O<sub>2</sub> hearts: treated for 20 min with 300 μM H<sub>2</sub>O<sub>2</sub> mixed in the perfusion medium. The selection of different concentrations of X, XO and





**Figure 8:** Schematic representation of the experimental design for hypoxia and/or glucose deprivation-reperfusion.



**Figure 9:** Schematic representation of the experimental design for perfusion with reactive oxygen species.

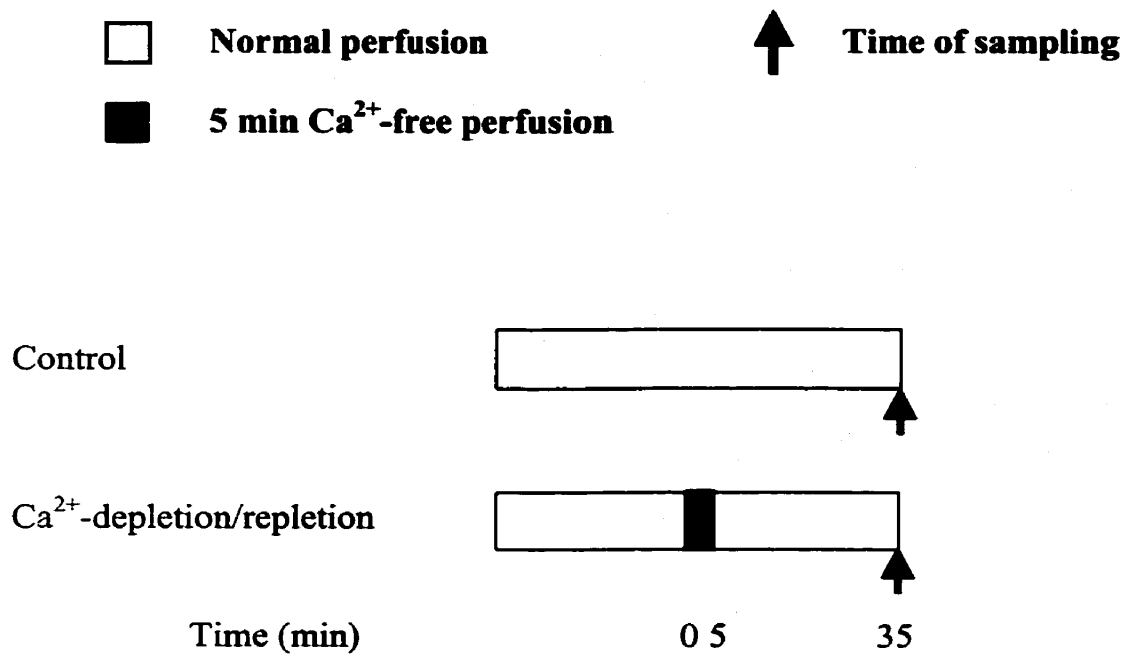
H<sub>2</sub>O<sub>2</sub> is based on our previous experience with these agents (175, 222).

## 6. Protocol for Ca<sup>2+</sup>-paradox

After a 30 min period of stabilization, hearts were divided into two groups (**Figure 10**): (a) control hearts (C): perfused for 35 min with normal K-H medium, (b) Ca<sup>2+</sup>-depleted/repleted hearts (Ca<sup>2+</sup>-paradox: CP): perfused for 5 min with Ca<sup>2+</sup>-free medium then reperfused for 30 min with K-H medium containing 1.25 mM Ca<sup>2+</sup>. The perfusion procedure was adapted according to our earlier study (251).

## 7. Protocol for ischemic-preconditioning

After a 30 min period of stabilization, hearts were divided into eight groups (**Figure 11, Panels A and B**): (a) control hearts (C): perfused for 90 min with normal K-H medium, (b) ischemic-preconditioning (IP: 3x5'I/5'R): exposed to 3 cycles of 5 min ischemia and 5 min reperfusion, (c) ischemic-reperfused hearts (IR): perfused for 30 min with normal K-H medium then exposed to 30 min of global ischemia followed by 30 min of reperfusion, (d) IP+IR hearts, (e) control hearts (C): perfused for 53 min with normal K-H medium, (f) ischemic-preconditioning (IP: 3x3'I/3'R): exposed to 3 cycles of 3 min ischemia and 3 min reperfusion, (g) Ca<sup>2+</sup>-depleted/repleted hearts (Ca<sup>2+</sup>-paradox: CP): perfused with normal K-H medium for 18 min followed by 5 min of Ca<sup>2+</sup>-free perfusion and 30



**Figure 10:** Schematic representation of the experimental design for Ca<sup>2+</sup>-paradox.



min of perfusion with K-H medium containing 1.25 mM  $\text{Ca}^{2+}$ , (h) IP+CP. Three cycles of IP whether for 3 or 5 min (3'I/3'R or 5'I/5'R) had similar effects on cardiac performance and the expression levels of SR genes in IR hearts. Likewise, similar results were obtained when CP hearts were preconditioned with 3 cycles of 3'I/3'R or 5'I/5'R. The perfusion protocols were similar to our previous reports (130, 130, 251).

## **8. SR membrane isolation**

Membrane fraction enriched with SR was isolated according to a previously described method (147). Briefly, the left ventricular tissue was pulverized and homogenized twice for 20 sec each at half maximal setting using a Polytron homogenizer (Brinkman, Westbury, NY). The homogenization buffer contained (in mM): 10  $\text{NaHCO}_3$ , 5  $\text{NaN}_3$ , 15 Tris-HCl, pH 6.8 and protease inhibitors (in  $\mu\text{M}$ ): 1 leupeptin, 1 pepstatin and 100 phenylmethyl-sulfonylfluoride. The homogenate was then centrifuged for 20 min at 9,500 rpm (Beckman, JA 20.0) and the supernatant obtained was further centrifuged for 45 min at 19,000 rpm (Beckman, JA 20). The pellet obtained was suspended in a buffer containing 0.6 M KCl, 20 mM Tris-HCl, pH 6.8, and centrifuged at the same speed and duration of the last step; the pellet thus obtained was suspended in a mixture of 0.25 M sucrose, 10 mM histidine, pH 7.0. All steps were performed at 4° C and the SR suspension was later used for various assays. The protein concentration of the SR preparations was measured by Lowry method (302). The purity of the membrane

preparation was determined by measuring the activities of marker enzymes according to methods described earlier (130). SR preparations employed in this study showed 3 to 5% cross contamination with other subcellular organelles; however, the degree of contamination was of equal extent for each of the control and experimental groups.

### **9. Determination of SR Ca<sup>2+</sup>-uptake**

Ca<sup>2+</sup>-uptake activity of the SR vesicles was determined by the procedure of Hawkins et al (303). The standard reaction mixture (total volume 250  $\mu$ L) contained (in mM): 50 Tris-maleate (pH 6.8), 5 NaN<sub>3</sub>, 5 ATP, 5 MgCl<sub>2</sub>, 120 KCl, 5 K-oxalate, 0.1 EGTA, 0.1 <sup>45</sup>CaCl<sub>2</sub> (12,000 cpm/nmol) and 0.25 ruthenium red. The concentration of free Ca<sup>2+</sup> in this medium determined by the program of Fabiato (304) was 8.2  $\mu$ M. Ruthenium red was added to inhibit the Ca<sup>2+</sup>-release channel activity under these conditions. The reaction was initiated by the addition of SR membranes (20  $\mu$ g) to the Ca<sup>2+</sup>-uptake reaction mixture and terminated after 1 min by filtering 200  $\mu$ L aliquot of the reaction mixture. The filters were washed with 5 ml washing buffer, dried at 60° C for 1 hr and counted in a beta liquid scintillation counter.

### **10. Determination of SR Ca<sup>2+</sup>-release**

Calcium release activity of the SR vesicles was measured by the procedure used by Temsah et al (147). In brief, the SR sample (31.25  $\mu$ g protein) was

suspended in a total volume of 625  $\mu\text{l}$  of loading buffer containing (in mM): 100 KCl, 5  $\text{MgCl}_2$ , 5 K-oxalate, 5  $\text{NaN}_3$  and 20 Tris-HCl (pH 6.8). After incubation with 10  $\mu\text{M}$   $^{45}\text{CaCl}_2$  and 5 mM ATP for 45 min at room temperature, 100  $\mu\text{l}$  of the reaction mixture was filtered for determination of basal value. At the 46 min  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release was carried out by adding 1 mM EGTA plus 1 mM  $\text{CaCl}_2$  to the reaction mixture. The reaction was terminated at 15 sec by Millipore filtration technique. Radioactivity in the filter was counted in 10 ml of scintillation fluid. The  $\text{Ca}^{2+}$ -induced- $\text{Ca}^{2+}$ -release was completely prevented (95 to 97%) by the treatment of SR preparations with 20  $\mu\text{M}$  ryanodine.

#### **11. SDS-PAGE and Western blot assay**

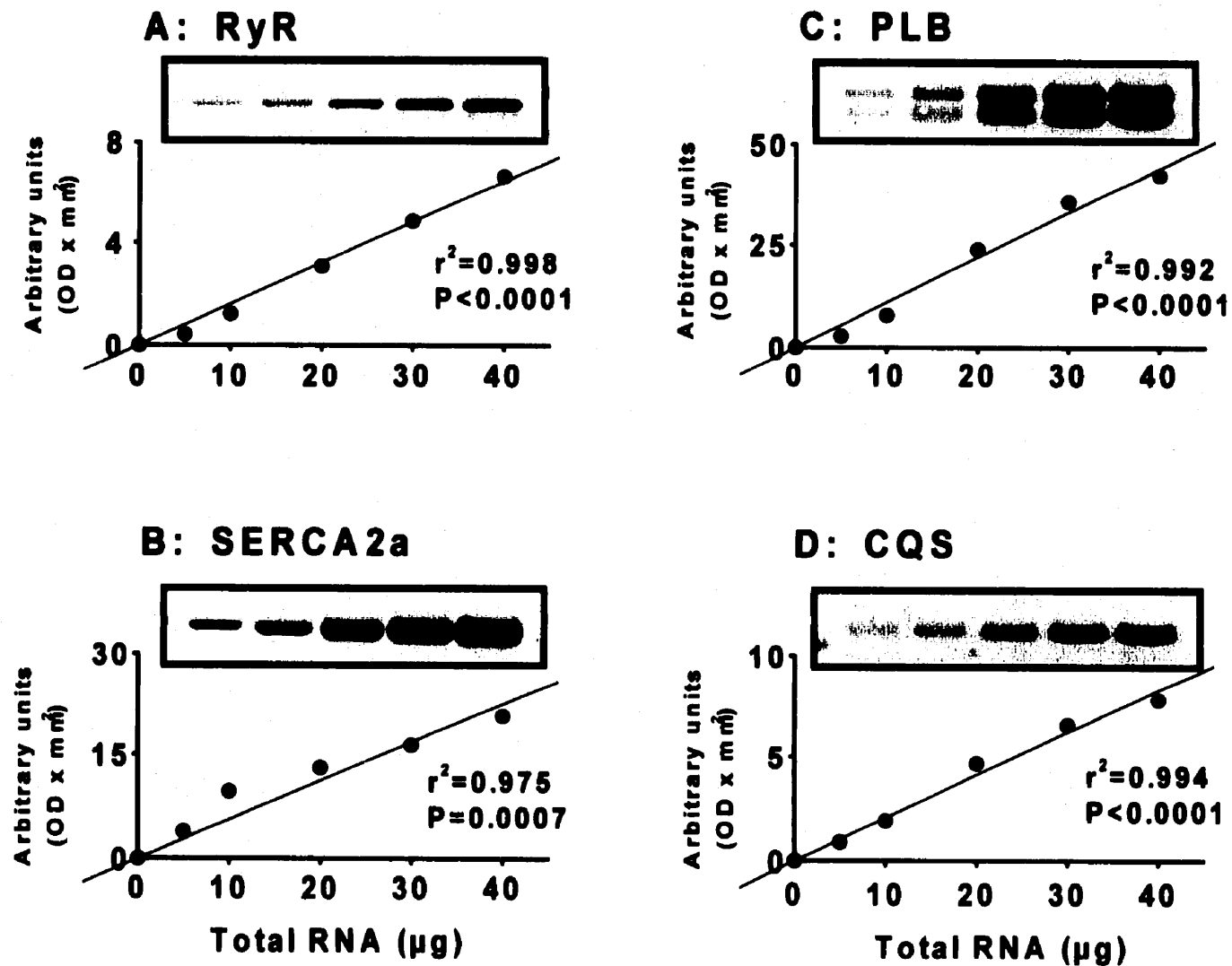
The relative amounts of RyR, SERCA2a and PLB were determined according to a previously described method (147). Protein samples (20  $\mu\text{g/ml}$ ) were suspended (1:1) with the Laemmli buffer containing: 0.1 M Tris-HCl (pH 6.8), 15% (w/v) sodium dodecyl sulphate (SDS), 15% glycerol, 8%  $\beta$ -mercaptoethanol and 0.002% bromophenol blue, and then denatured by boiling for 10 min. SR samples (20  $\mu\text{g}$  protein) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by running 5% mini gel for RyR, 10% for SERCA2a and 15% for PLB. The protein bands for SERCA2a and PLB were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA, USA) while that for RyR was transferred to nitrocellulose membrane at 100 V. The transfer buffer contained 25 mM Tris-HCl,



192 mM glycerine and 4% methanol (v/v). The transferred membranes were shaken overnight in blocking buffer (TBS, 10 mM Tris, 150 mM NaCl and 5% fat-free powdered milk) at 4° C. The membranes were then incubated for 1 hr at room temperature in monoclonal anti-SERCA2a (1:1400) (Affinity Bioreagents Inc., Golden, CO, USA), monoclonal anti-ryanodine receptor (1:1400) and monoclonal anti-phospholamban (1:2000) (Upstate Biotechnology, Lake Placid, NY, USA). The membranes were incubated for 1 hr with a secondary antibody. An anti-mouse peroxidase-linked IgG (1:5000, Amersham Life Science, Oakville, ON) was used as a secondary antibody for SERCA2a and PLB whereas anti-mouse biotinylated IgG (1:2500, Amersham Life Science, Oakville, ON) was used for RyR. The RyR membranes were then incubated with streptavidin-conjugated horseradish peroxidase (1:5000, Amersham Life Science, Oakville, ON) in TBST for 30 min at room temperature. The blots were rinsed in the TBST buffer 3 times (15 min each time) between each of the preceding steps. Antibody-antigen complexes in all membranes were detected by the chemiluminescence ECL kit (Amersham Corporation, Arlington Heights, IL, USA) and the protein bands were then visualized on Hyperfilm-ECL (Amersham Corporation, Arlington Heights, IL, USA). An Imaging Densitometer was used to scan the protein bands and quantified using the Image Analysis Software Version 1.0. Equal protein loading was checked in every experiment by staining the membrane with Ponceau S before immunoblotting and with Coomassie Brilliant Blue at the end of the experiment.

## 12. Northern blot analysis and molecular probes

Total RNA was extracted from ventricular tissue by the guanidinium thiocyanate method (305). The linearity of the procedure used for RNA isolation and quantification of RyR, SERCA2a, PLB and CQS is shown in **Figure 12** (Panels A-D). Samples normalized to 5, 10, 20, 30 and 40  $\mu\text{g}$  of total RNA in sterile distilled water containing 0.2% DEPC were denatured at 65° C for 10 min and electrophoresed in a 1.2% agarose /formaldehyde gel containing 1 M formaldehyde. Twenty  $\mu\text{g}$  of total RNA were used in the rest of the study since it is in the linear range. The fractionated mRNA transcripts were transferred to a charge-modified nylon filter (NYTRAN Maximum Strength Plus, Schleicher and Schuell, Keene, NH, USA) for 24 hours. The membrane was then UV cross-linked (UV Stratalinker 2400, Stratagene). Blots were prehybridized at 42° C overnight using INNOVA 4080 incubator oscillating at a rate of 65 rpm. Labeled random primed cDNA or oligonucleotide probes were added to the prehybridization solution and left overnight under the same conditions. The membranes were washed with 1 X standard saline citrate and 0.1% SDS at room temperature and exposed to Kodak X-Omat-AR film using intensifying screen at -70° C. The radiolabeled mRNA bands were scanned using a densitometer GS-670 (Bio-Rad Company, Mississauga, ON, Canada) and quantified with the Image Analysis Software Version 1.0. The optical density of each band was divided by that of the 18S band for normalization as an internal standard and the relative levels were



**Figure 12:** Relationship between the amount of RNA sample and the intensity of mRNA blots. RyR (Panel A), SERCA2a (Panel B), PLB (Panel C), and CQS (Panel D) mRNA levels in heart. Regression-squared ( $r^2$ ) and probability (P) values are shown in each scattergram.

calculated as percentage of the mean value of the corresponding controls. The inserts were separated from recombinant plasmids and used as probes. RyR: a 2.2 kb cDNA fragment from the rabbit cardiac ryanodine receptor (courtesy of Dr. A.K. Grover, McMaster University, Hamilton, ON, Canada). SERCA2a: a 0.762-kb cDNA fragment from the rabbit heart  $\text{Ca}^{2+}$ -pump ATPase (courtesy of Dr. A.K. Grover, McMaster University, Hamilton, ON, Canada). PLB: a 0.153 kb cDNA fragment from the rabbit heart (courtesy of Dr. D.H. MacLennan, University of Toronto, Toronto, Canada). CQS was a 2.5 kb cDNA fragment from the rabbit heart (courtesy of Dr. A. Zilberman, University of Cincinnati, Cincinnati, USA). 18S was a 24 base oligonucleotide probe (5'-ACGGTATCTGATCGTCTTCGAA CC-3') of the rat ribosomal RNA and was used as an internal standard to account for differences in nucleic acid loading and/or transfer. The cDNA used to hybridize specific mRNA transcripts were prepared and autoradiographed using a Random Primer DNA Labeling system (New England Nuclear, Boston, MA) radiolabelled with  $\alpha$ - $^{32}\text{P}$ -dCTP.

### **13. Statistical analysis**

All results were expressed as mean $\pm$ SE and were statistically evaluated by Analysis of Variance (ANOVA) test followed by the Student's unpaired t-test for multiple comparisons. Linear regression test was used for the linearity study.  $P < 0.05$  was considered the threshold for statistical significance between the control and the experimental groups.

## IV. RESULTS

### 1. Effect of ischemia-reperfusion with and without SOD plus CAT

#### a. Alterations in cardiac performance and SR function

Hearts subjected to global ischemia for 30 min showed a significant decrease in LVDP +dP/dt and -dP/dt, as well as a marked increase in LVEDP (7 fold) (Table 4). Reperfusion of ischemic hearts for 60 min recovered the contractile function as represented by LVDP, +dP/dt and -dP/dt by 22 to 28% of the control values, whereas the LVEDP was increased about 12 fold. The recovery of contractile activity in IR hearts was markedly improved by SOD plus CAT treatment; this was reflected by 78 to 84% recovery of LVDP, +dP/dt and -dP/dt as well as about 4 fold increase in LVEDP in comparison to the control heart preparation. In the ischemic and IR hearts, SR  $\text{Ca}^{2+}$ -uptake and  $\text{Ca}^{2+}$ -release activities were significantly reduced (Table 5). Treatment of hearts with SOD plus CAT showed a complete and partial recovery of changes in  $\text{Ca}^{2+}$ -uptake and release activities, respectively.

#### b. Alterations in SR protein levels and mRNA expression

The protein content of RyR and SERCA2a decreased in the ischemic heart by 50% and 27%, respectively, when compared to controls, while PLB content did not change (Figure 13, Panels A and B). Reperfusion after ischemia decreased the protein content of RyR (by 66%), SERCA2a (by 72%), and PLB (by 14%).

**Table 4: Effect of ischemia-reperfusion with and without SOD plus CAT treatment on cardiac performance.**

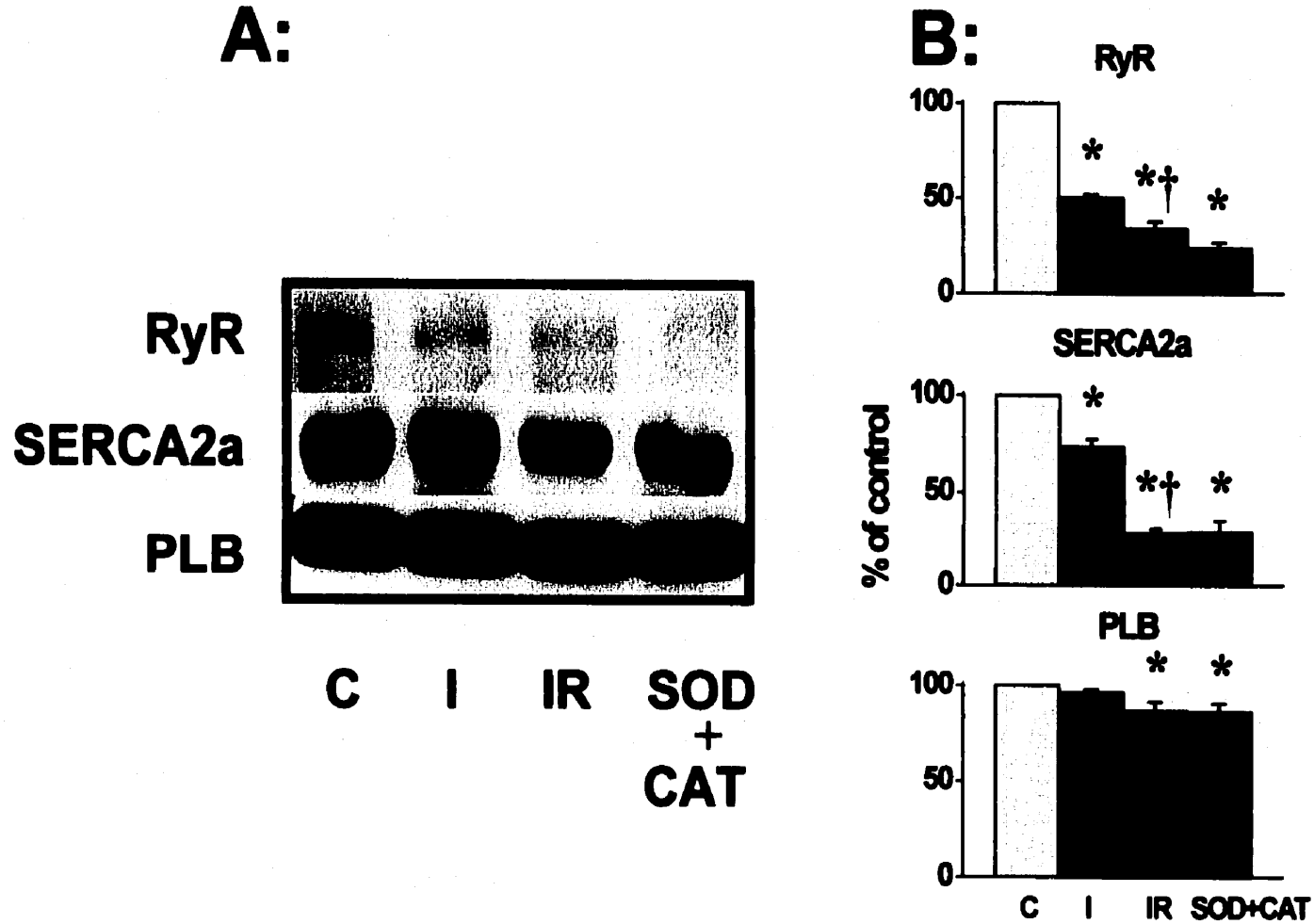
Group	LVDP	LVEDP	+dP/dt	-dP/dt
	mmHg		mmHg/sec	
Control	84.1±3.8	10.1±0.4	2590±74	1630±40
Ischemia	2.3±0.1*	43.8±3.1*	108±19*	51±4.0*
Ischemia-reperfusion	23.7±2.0*†	124±1.9*†	564±29*†	408±10*†
SOD plus CAT	70.4±2.3#	40.4±2.5*#	2014±25*#	1324±21#

Values are mean±SE of 6 separate experiments. \*P < 0.05 vs. control, † vs. ischemia, # vs. ischemia-reperfusion.

**Table 5: Effect of ischemia-reperfusion with and without SOD plus CAT treatment on SR function.**

<b>Group</b>	<b>Ca<sup>2+</sup>-uptake (nmol <sup>45</sup>Ca/mg/min)</b>	<b>Ca<sup>2+</sup>-release (nmol <sup>45</sup>Ca/mg/15 sec)</b>
<b>Control</b>	56.1±2.1	9.63±1.47
<b>Ischemia</b>	19.3±10.9*	2.86±1.05*
<b>Ischemia-reperfusion</b>	28.1±0.7*	2.81±0.34*
<b>SOD plus CAT</b>	53.8±2.3#	5.25±0.64*#

Values are mean±SE of 6 separate experiments. \*P < 0.05 vs. control, # vs. ischemia-reperfusion.



**Figure 13:** Effect of ischemia-reperfusion on SR protein levels. Panel A shows the autoradiogram and Panel B depicts the analysis of the SR gene expression in control (C), ischemia (I), ischemia-reperfusion (IR), and IR hearts treated with SOD plus CAT. Values are mean  $\pm$ SE of 6 separate experiments. \*  $P < 0.05$  vs. C, † vs. I.



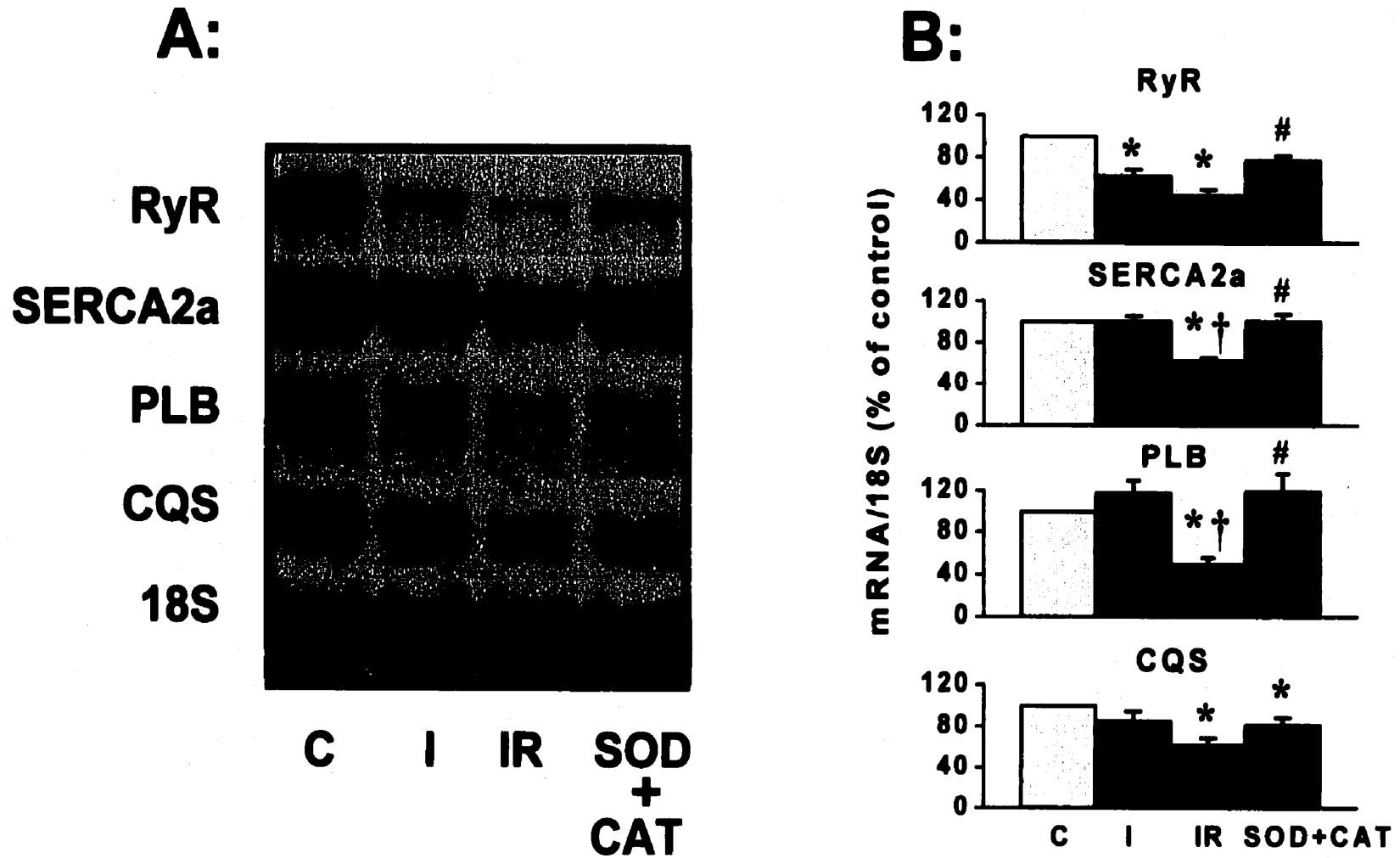
Treatment with SOD plus CAT did not recover the protein content of RyR, SERCA2a or PLB.

Northern blot analysis showed that ischemia reduced the mRNA levels of RyR by 33.5%, SERCA2a by 16% and CQS by 21% in comparison to the controls whereas PLB mRNA level remained unchanged (**Figure 14, Panels A and B**). Reperfusion after ischemia significantly decreased the transcript levels of RyR (by 56%), SERCA2a (by 37.7%), PLB (by 28.7%) and CQS (by 46.4%) in comparison to controls. When compared with IR, treatment with SOD plus CAT improved the mRNA levels of RyR (by 33.7%), SERCA2a (by 24.5%), PLB (by 35.2%) and CQS (by 27.4%).

## **2. Effect of $\beta$ -adrenoceptor blockers on ischemic-reperfused hearts**

### **a. Alterations in cardiac performance and SR function**

Reperfusion for 60 min after ischemia showed 28% recovery in LVDP, 19% recovery in +dP/dt and 19% recovery in -dP/dt. On the other hand, LVEDP in the IR hearts increased by 7.7-fold over the control value (**Table 6**). IR hearts treated with 10  $\mu$ M atenolol demonstrated a significant improvement in cardiac performance as reflected by 45% recovery in LVDP, 44 % recovery in the +dP/dt and 64% recovery in -dP/dt; the LVEDP was significantly lower by this treatment but was still 5.2-fold higher than the control. Treatment with 10  $\mu$ M propranolol also showed a significant improvement in cardiac function, as the recovery in LVDP, +dP/dt and -dP/dt was 67, 57, and 79% in comparison to pre-ischemic



**Figure 14:** Effect of ischemia-reperfusion on SR mRNA expression. Panel A shows the autoradiogram and Panel B depicts the analysis of the SR mRNA expression in control (C), ischemia (I), ischemia-reperfusion (IR), and IR hearts treated with SOD plus CAT. Values are mean  $\pm$ SE of 6 separate experiments. \*  $P < 0.05$  vs. C, † vs. I, # vs. IR.

**Table 6: Effect of  $\beta$ -adrenoceptor blockers on cardiac performance of ischemic-reperfused hearts.**

Group	LVDP	LVEDP	+dP/dt	-dP/dt
	mmHg		mmHg/sec	
<b>Control</b>	85.0 $\pm$ 4.2	8.4 $\pm$ 0.6	2399 $\pm$ 61	1753 $\pm$ 39
<b>Ischemia-reperfusion</b>	23.8 $\pm$ 3.0*	64.7 $\pm$ 6.1*	456 $\pm$ 28*	333 $\pm$ 11*
<b>Atenolol treated IR hearts</b>	36.6 $\pm$ 1.2*†	43.7 $\pm$ 2.0*†	1056 $\pm$ 32*†	1122 $\pm$ 23*†
<b>Propranolol treated IR hearts</b>	56.7 $\pm$ 3.1*†#	28.4 $\pm$ 1.8*†#	1367 $\pm$ 32*†#	1384 $\pm$ 18*†#

Values are mean $\pm$ SE of 6 separate experiments. \*P < 0.05 vs. control, † vs. ischemia-reperfusion, # vs. atenolol.

values, respectively. Although the level of LVEDP in propranolol-treated hearts was 3.6-fold higher than the control, it was significantly less than that in the IR group. SR  $\text{Ca}^{2+}$ -uptake and  $\text{Ca}^{2+}$ -release were significantly reduced in IR hearts (Table 7) while treatment with 10  $\mu\text{M}$  atenolol or propranolol significantly improved both activities.

**b. Alterations in SR protein levels and gene expression**

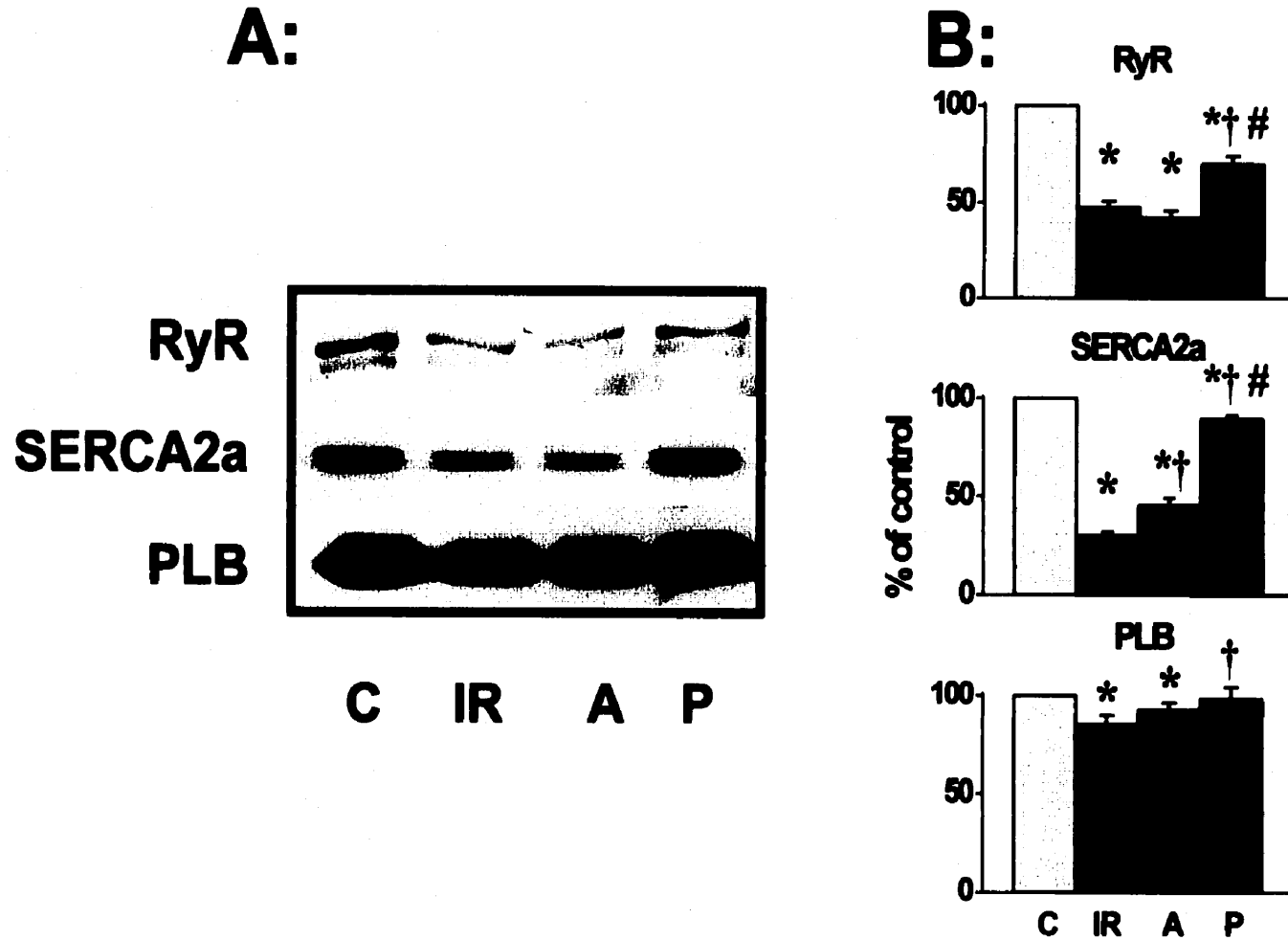
In IR hearts the protein levels of RyR, SERCA2a and PLB were depressed by 55%, 70% and 15% from the control levels, respectively (Figure 15, Panels A and B). Hearts treated with atenolol showed a slight, but significant, recovery only in SERCA2a protein levels (by 15%) when compared with IR protein levels. On the other hand, propranolol-treated hearts showed a significant recovery in RyR (by 15%), SERCA2a (by 60%) and PLB (by 15%) from IR levels.

The analysis of the autoradiograms revealed that IR significantly decreased the levels of mRNA for RyR by 89%, SERCA2a by 61%, PLB by 58% and CQS by 48% when compared to the control (Figure 16, Panels A and B). Treatment with atenolol showed improvement over IR with respect to mRNA levels for RyR, SERCA2a, PLB and CQS by 26, 25, 20 and 24%, respectively. Propranolol also significantly improved the mRNA levels for RyR by 40%, SERCA2a by 39%, PLB by 23% and CQS by 29% when compared with IR group.

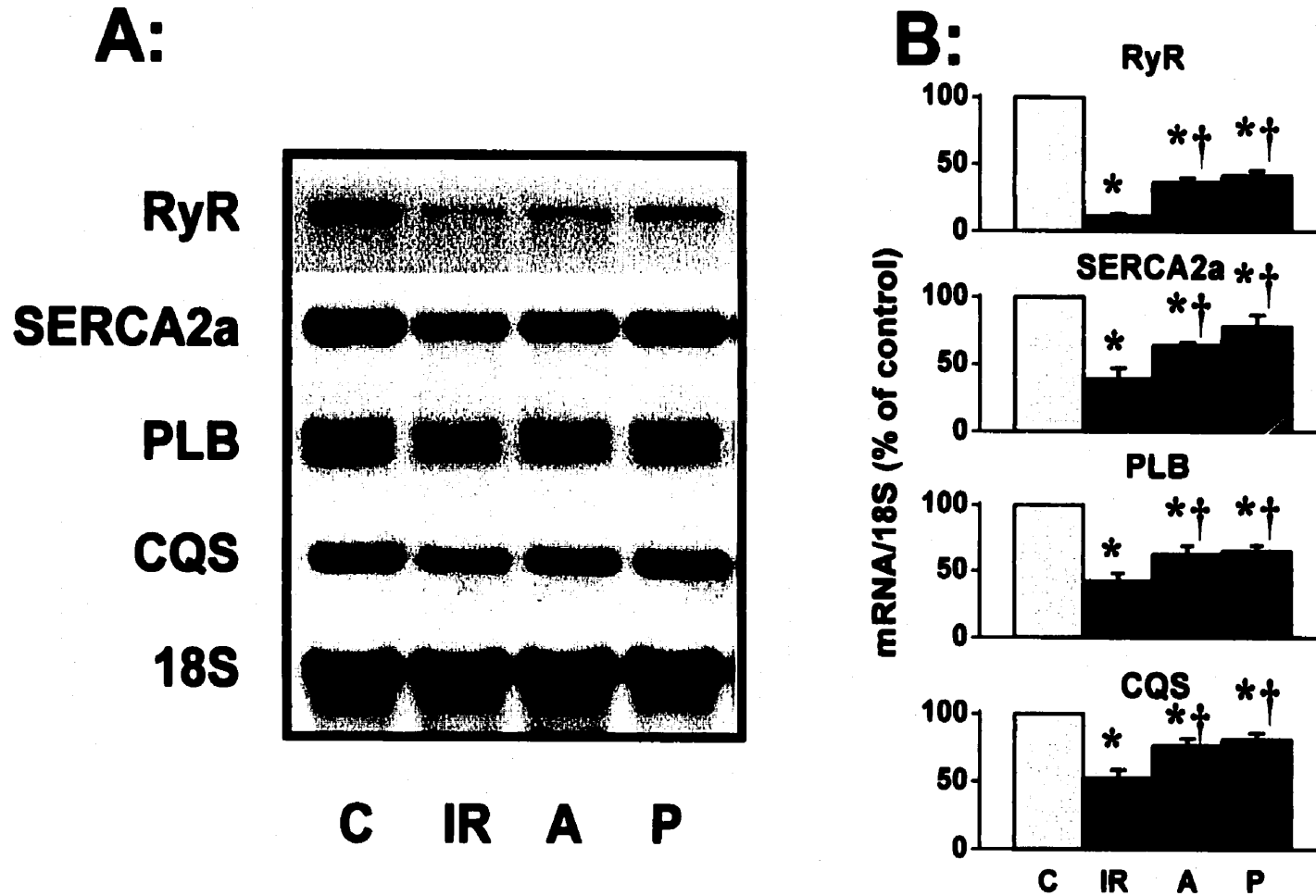
**Table 7: Effect of  $\beta$ -adrenoceptor blockers on SR function of ischemic-reperfused hearts.**

<b>Group</b>	<b>Ca<sup>2+</sup>-uptake (nmol <sup>45</sup>Ca/mg/min)</b>	<b>Ca<sup>2+</sup>-release (nmol <sup>45</sup>Ca/mg/15 sec)</b>
<b>Control</b>	34.6 ± 5.6	9.7 ± 1.2
<b>IR</b>	17.0 ± 2.6*	4.5 ± 0.3*
<b>Atenolol treated IR hearts</b>	28.4 ± 2.5	7.1 ± 0.9
<b>Propranolol treated IR hearts</b>	33.8 ± 6.0	8.9 ± 1.0

Values are mean±SE of 6 separate experiments. IR, ischemia-reperfusion. \*P < 0.05 vs. control.



**Figure 15:** Effect of  $\beta$ -adrenoceptor blockade on SR protein levels in ischemic-reperfused hearts. Panel A shows the autoradiogram and Panel B depicts the analysis of the SR gene expression in control (C), ischemia-reperfusion (IR), IR hearts treated with atenolol (A) and propranolol (P). Values are mean  $\pm$ SE of 6 separate experiments. \*  $P < 0.05$  vs. C, † vs. IR, # vs. atenolol.



**Figure 16:** Effect of  $\beta$ -adrenoceptor blockade on SR mRNA expression in ischemic-reperfused hearts. Panel A shows the autoradiogram and Panel B depicts the analysis of the SR mRNA expression in control (C), ischemia (I), ischemia-reperfusion (IR), and IR hearts treated with atenolol (A) and propranolol (P). Values are mean  $\pm$ SE of 6 separate experiments. \*  $P < 0.05$  vs. C, † vs. IR.

### **3. Effect of hypoxia and/or glucose deprivation-reperfusion**

#### **a. Alterations in cardiac performance and SR function**

Hypoxic or glucose-deprived hearts showed a significant decrease in LVDP (by 44-61%), +dP/dt (by 50-55%) and -dP/dt (63-82%) and a marked increase in LVEDP (2-4 fold) (Table 8). While hypoxic hearts completely recovered upon perfusion for 60 min with control medium, glucose-deprived hearts showed partial recovery. A more drastic decrease in LVDP (by 91%) and a greater increase in LVEDP (11 fold) were observed after 30 min of perfusion with hypoxic and glucose-free medium; these alterations persisted after 60 min of perfusion with control medium although there was a tendency towards recovery. Hypoxia or glucose deprivation followed by reperfusion had no effect on the SR function whereas hypoxia plus glucose deprivation followed by reperfusion reduced the  $\text{Ca}^{2+}$ -uptake and release activities (Table 9). Since the objective of the study was to examine the role of hypoxia and glucose deprivation in the IR-induced changes, the samples were not collected after the deprivation period and therefore the SR  $\text{Ca}^{2+}$ -uptake and release activities were not determined at the end of that phase.

#### **b. Alterations in SR protein levels and mRNA expression**

There was no change in the protein content in hypoxia and/or glucose deprived-reperfused hearts followed by reperfusion (Figure 17, Panels A and B) except for a significant increase in SERCA2a mRNA in the glucose deprived-reperfused hearts.



**Table 8: Effect hypoxia and/or glucose deprivation-reperfusion on cardiac performance.**

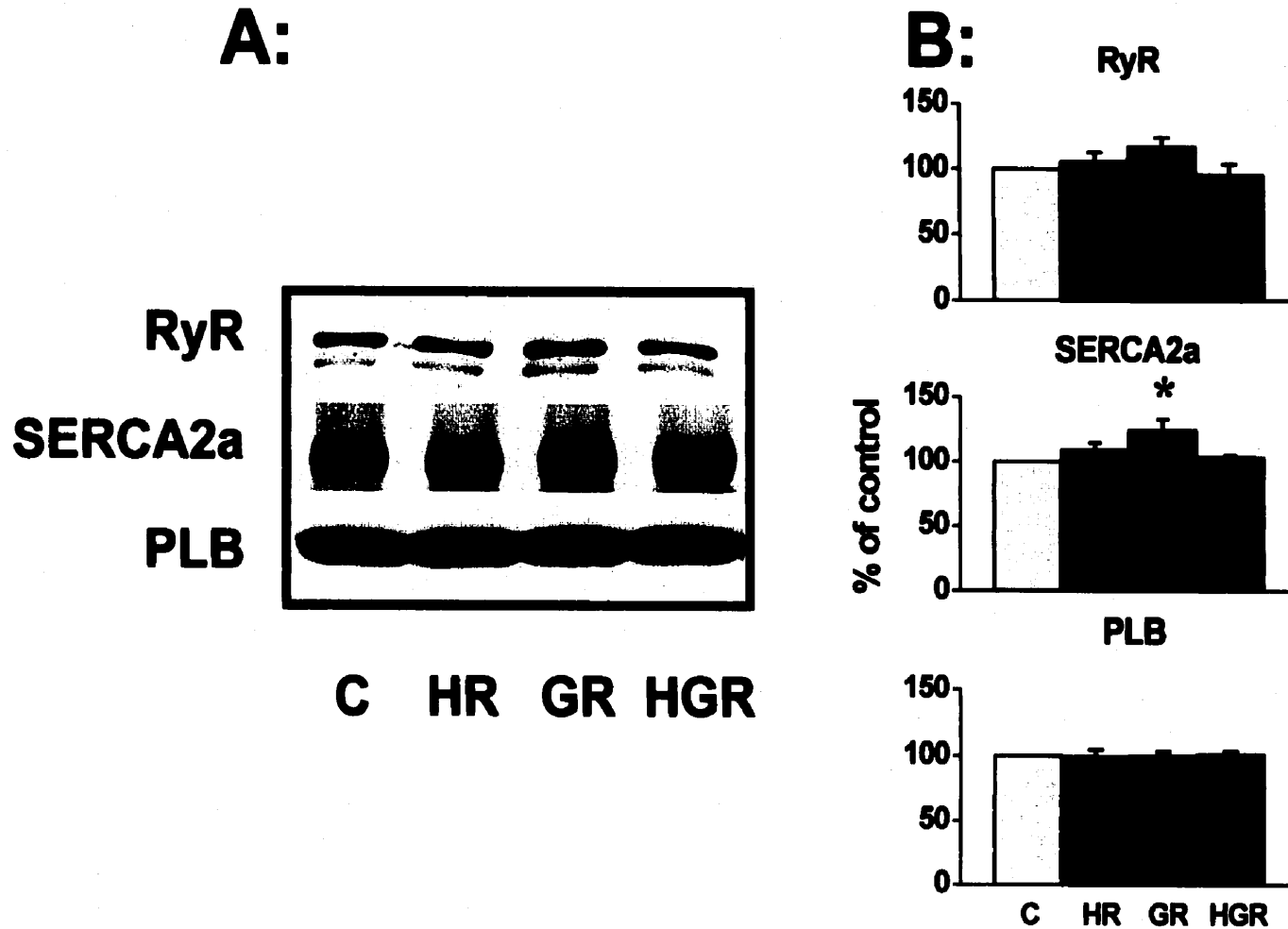
Group	LVDP	LVEDP	+dP/dt	-dP/dt
	mmHg		mmHg/sec	
<b>Control</b>	86.6±2.9	7.6±1.0	2677±80	1569±70
<b>Hypoxia</b>				
30 min hypoxia	38.4±1.7*	16.4±1.5*	1331±60*	992±44*
30 min hypoxia and 60 min control perfusion	74.0±2.7	5.6±1.7	2425±150*	1533±88
<b>Glucose deprivation</b>				
30 min glucose-free	52.6±5.8*	31.9±9.9*	1462±351*	1265±183*
30 min glucose-free and 60 min control perfusion	74.1±4.1*	24.1±7.7*	2268±128*	1282±114*
<b>Hypoxia and glucose deprivation</b>				
30 min hypoxia and glucose-free	7.9±2.7*	84.8±9.1*	115±15*	103±8.1*
30 min hypoxia and glucose-free and 60 min control perfusion	38.5±6.4*	44.0±12.3*	1194±261*	783±122*

Values are mean±SE of 6 separate experiments. \*P < 0.05 vs. control.

**Table 9: Effect of hypoxia and/or glucose deprivation on SR function.**

<b>Group</b>	<b>Ca<sup>2+</sup>-uptake (nmol <sup>45</sup>Ca/mg/min)</b>	<b>Ca<sup>2+</sup>-release (nmol <sup>45</sup>Ca/mg/15 sec)</b>
<b>Control</b>	53.4±1.0	8.3±0.4
<b>Hypoxia-reperfusion</b>	55.9±4.4	6.6±0.7
<b>Glucose deprivation-reperfusion</b>	57.2±4.2	7.2±1.0
<b>Hypoxia and glucose deprivation -reperfusion</b>	39.8±1.4*	3.0±0.9*

Values are mean±SE of 6 separate experiments. \*P < 0.05 vs. control.



**Figure 17:** Effect of hypoxia and/or glucose deprivation-reperfusion on SR protein levels. Panel A shows the autoradiogram and Panel B depicts the analysis of the SR gene expression in control (C), hypoxia-reperfusion (HR), glucose deprivation-reperfusion (GR), and hypoxia glucose deprivation-reperfusion (HGR). Values are mean  $\pm$ SE of 6 separate experiments. \*  $P < 0.05$  vs. C.

When the hypoxic or glucose-deprived hearts were perfused with normal oxygenated medium for 60 min, mRNA levels for SR proteins were not different from the control values (**Figure 18, Panels A and B**). On the other hand, perfusion of hearts deprived of both glucose and oxygen showed a significant decrease in the mRNA levels of RyR (by 49%), SERCA2a (by 35%), PLB (by 31%) and CQS (by 29%).

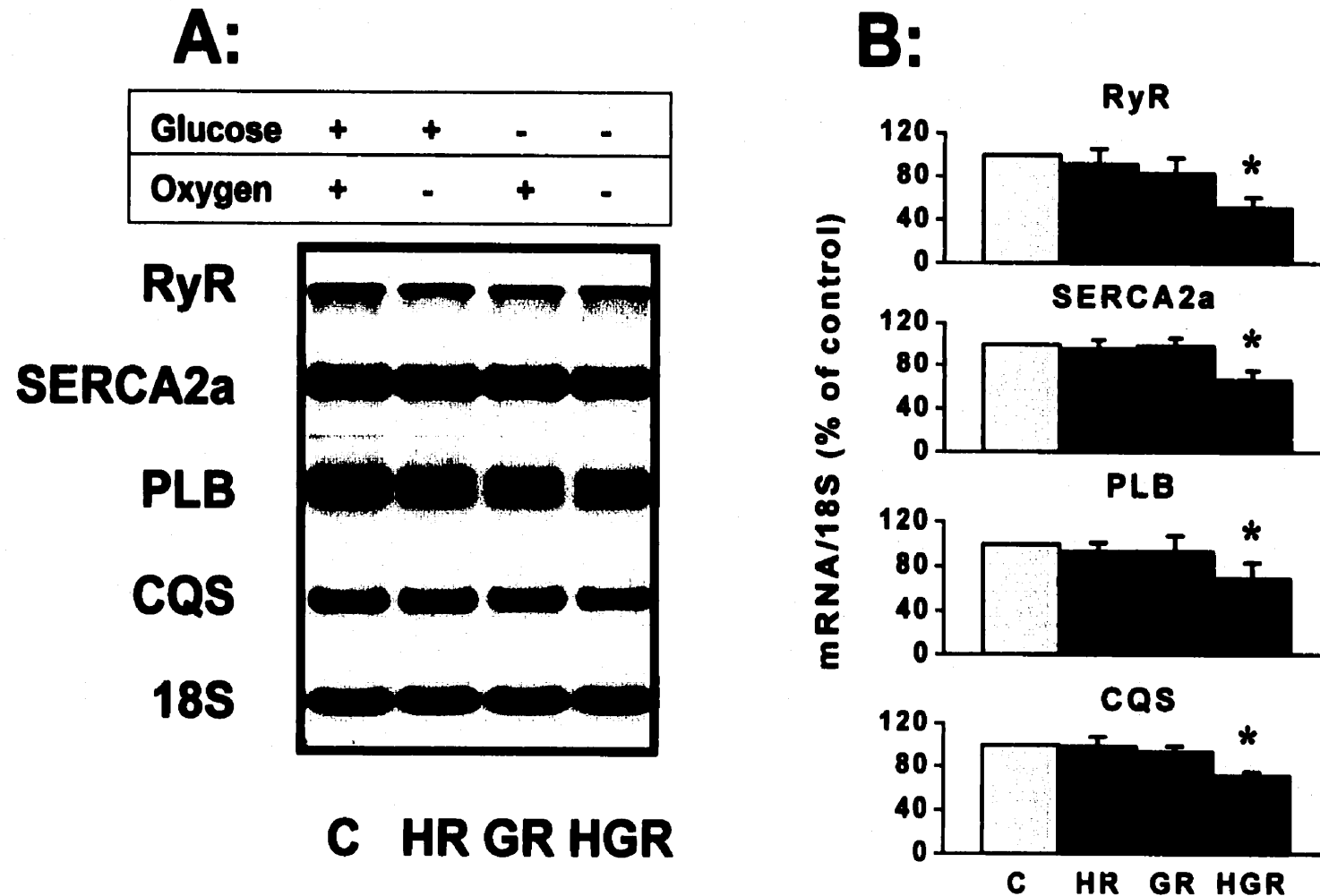
#### **4. Effect of reactive oxygen species**

##### **a. Alterations in cardiac performance and SR function**

The LVDP decreased to 15% in X plus XO and to 28% in H<sub>2</sub>O<sub>2</sub> perfused hearts when compared with control hearts (**Table 10**). The LVEDP increased by 10 and 6.6 fold in X plus XO and H<sub>2</sub>O<sub>2</sub> perfused hearts, respectively. Depressed cardiac function was accompanied by a marked decrease in the rate of contraction and relaxation. X plus XO decreased +dP/dt by 17 fold and -dP/dt by 11 fold. H<sub>2</sub>O<sub>2</sub> decreased +dP/dt and -dP/dt by 3.7 and 5.8 fold, respectively. In comparison to control values, SR Ca<sup>2+</sup>-uptake and release activities decreased significantly when hearts were perfused with either X plus XO or H<sub>2</sub>O<sub>2</sub> (**Table 11**).

##### **b. Alterations in SR protein levels and mRNA expression**

X plus XO reduced the protein levels of RyR, SERCA2a and PLB by 45%, 69%, and 30%, respectively, in comparison to control values (**Figure 19, Panels A and B**), while H<sub>2</sub>O<sub>2</sub> depressed only the SERCA2a protein content by 42%.



**Figure 18:** Effect of hypoxia and/or glucose deprivation-reperfusion on SR mRNA expression. Panel A shows the autoradiogram and Panel B depicts the analysis of the SR mRNA expression in control (C), hypoxia-reperfusion (HR), glucose deprivation-reperfusion (GR), and hypoxia glucose deprivation-reperfusion (HGR). Values are mean  $\pm$ SE of 6 separate experiments. \*  $P < 0.05$  vs. C.

**Table 10: Effect of reactive oxygen species on cardiac performance.**

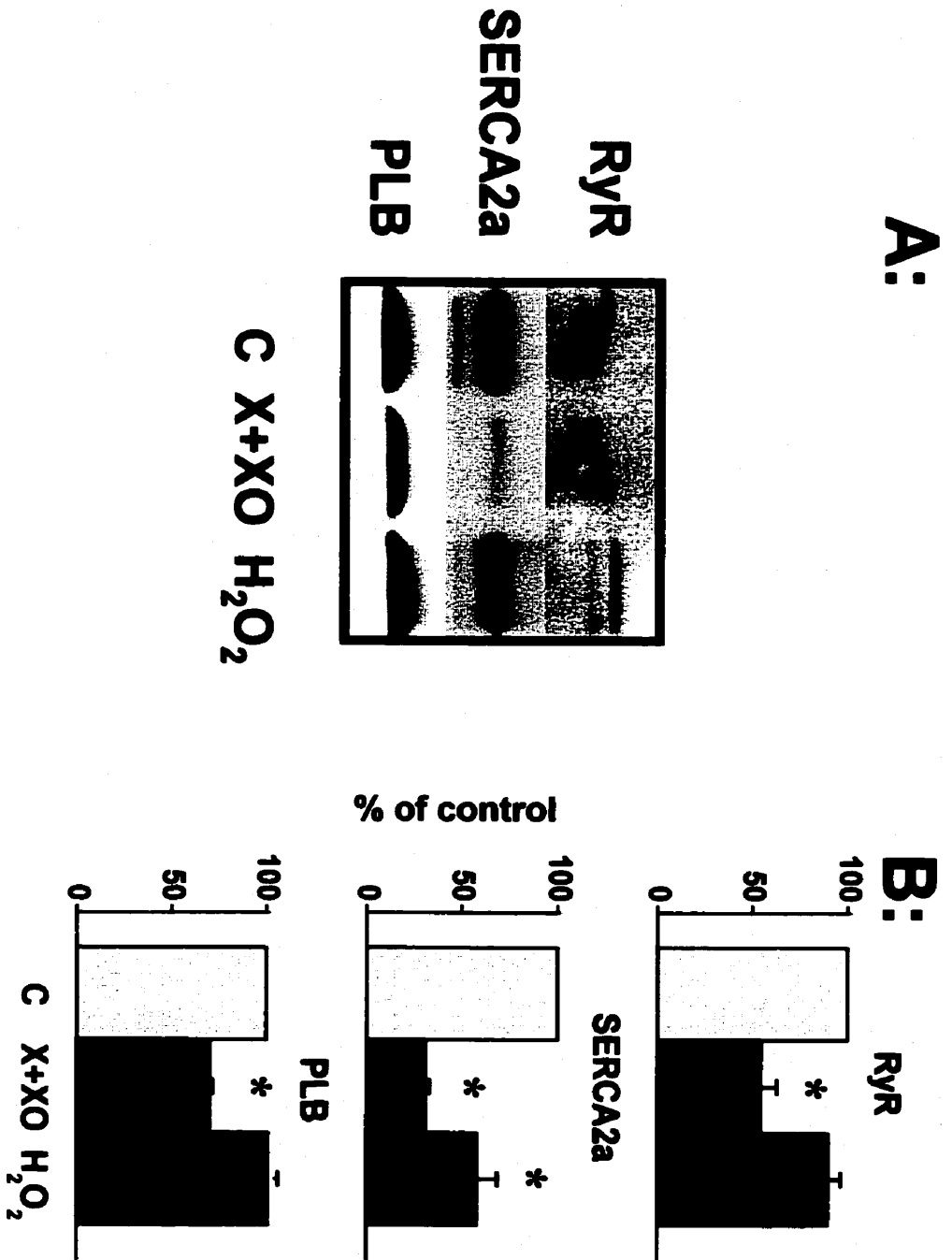
Group	LVDP	LVEDP	+dP/dt	-dP/dt
	mmHg		mmHg/sec	
Control	84.8±8.0	8.0±0.7	2661±179	1671±80
X plus XO	13.1±2.3*	79.3±12.8*	157±40*	152±25*
H <sub>2</sub> O <sub>2</sub>	23.9±1.8*	52.8±10.7*	725±81*	288±51*

Values are mean±SE of 6 separate experiments. \*P < 0.05 vs. control.

**Table 11: Effect of reactive oxygen species on SR function.**

<b>Group</b>	<b>Ca<sup>2+</sup>-uptake (nmol <sup>45</sup>Ca/mg/min)</b>	<b>Ca<sup>2+</sup>-release (nmol <sup>45</sup>Ca/mg/15 sec)</b>
<b>Control</b>	49.2±1.1	10.14±1.95
<b>X plus XO</b>	16.0±1.9*	1.5±0.07*
<b>H<sub>2</sub>O<sub>2</sub></b>	23.9±2.2*	2.32±0.12*

Values are mean±SE of 6 separate experiments. \*P < 0.05 vs. control.



**Figure 19:** Effect of reactive oxygen species on SR protein levels. Panel A shows the autoradiogram and Panel B depicts the analysis of the SR gene expression in control (C), X plus XO, and H<sub>2</sub>O<sub>2</sub> treated hearts. Values are mean  $\pm$ SE of 6 separate experiments. \* P<0.05 vs. C.



Hearts perfused with reactive oxygen species exhibited a depression in mRNA levels (**Figure 20, Panels A and B**) as in IR hearts. Perfusion with X plus XO or H<sub>2</sub>O<sub>2</sub> significantly decreased the amounts of RyR mRNA by 78-83%, SERCA2a mRNA by 53-55 %, PLB mRNA by 51-72%, and CQS mRNA by 52-55% in comparison to controls.

## 5. Effect of Ca<sup>2+</sup>-paradox

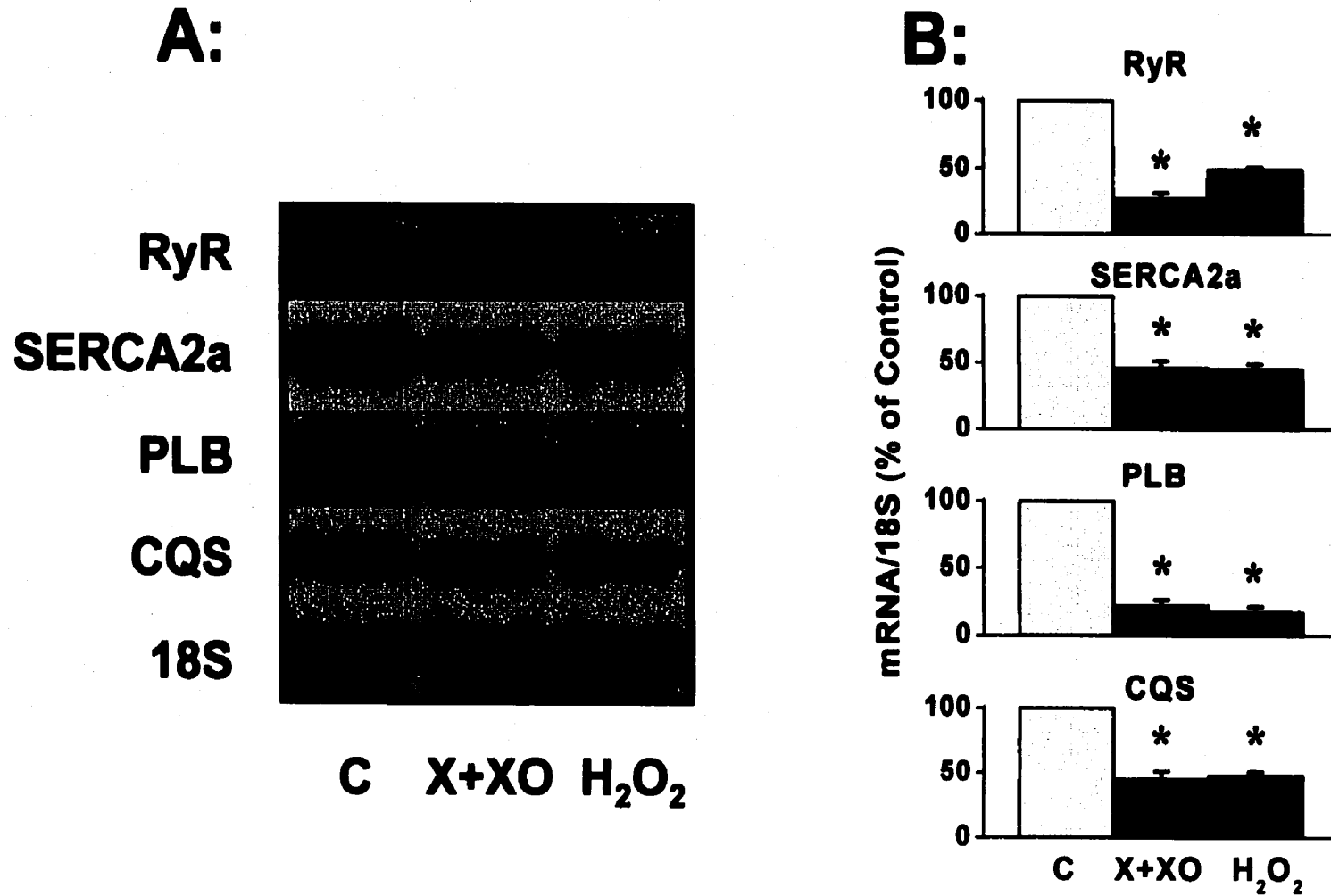
### a. Alterations in cardiac performance and SR function

Ca<sup>2+</sup>-depleted hearts failed to contract upon perfusion with Ca<sup>2+</sup>-free medium for 5 min; however, there was a significant increase in LVEDP (data not shown). The data in **Table 12** show that LVDP, +dP/dt and -dP/dt in the Ca<sup>2+</sup>-depleted hearts did not recover upon 30 min of reperfusion (Ca<sup>2+</sup>-repletion) with control medium whereas LVEDP was markedly augmented upon reperfusion. **Table 13** shows that the SR Ca<sup>2+</sup>-uptake and Ca<sup>2+</sup>-release activities were also depressed in these hearts in comparison to controls.

### b. Alterations in SR protein levels and mRNA expression

Ca<sup>2+</sup>-paradox reduced the protein levels of RyR, SERCA2 and PLB by 94.6, 96.8, and 44.7%, respectively (**Figure 21, Panels A and B**).

Ca<sup>2+</sup>-depletion/repletion, which is associated with a marked increase in the [Ca<sup>2+</sup>]<sub>i</sub> in the myocardium, decreased the RyR, SERCA2a, PLB and CQS mRNA by 65, 85, 60, and 75%, respectively (**Figure 22, Panels A and B**).



**Figure 20:** Effect of reactive oxygen species on SR mRNA expression. Panel A shows the autoradiogram and Panel B depicts the analysis of the SR mRNA expression in control (C), X plus XO, and H<sub>2</sub>O<sub>2</sub> treated hearts. Values are mean  $\pm$ SE of 6 separate experiments. \* P<0.05 vs. C.

**Table 12: Effect of Ca<sup>2+</sup>-paradox on cardiac performance.**

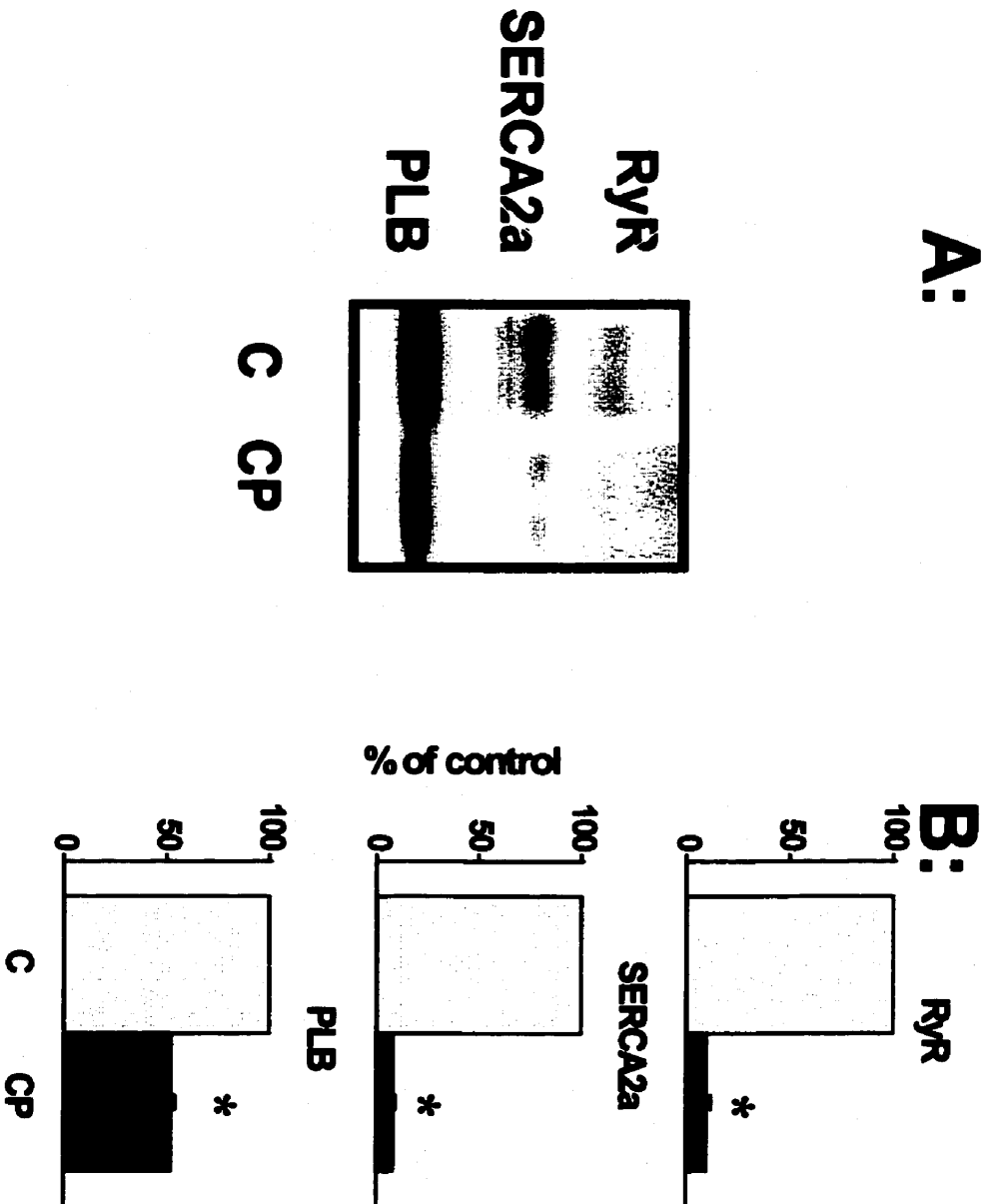
Group	LVDP	LVEDP	+dP/dt	-dP/dt
	mmHg		mmHg/sec	
Control	83.4±3.0	7.9±1.5	2741±79	1694±74
Ca <sup>2+</sup> -depletion/repletion	4.0±0.9*	74.9±6.2*	320±20*	384±18*

Values are mean±SE of 6 separate experiments. \*P < 0.05 vs. control.

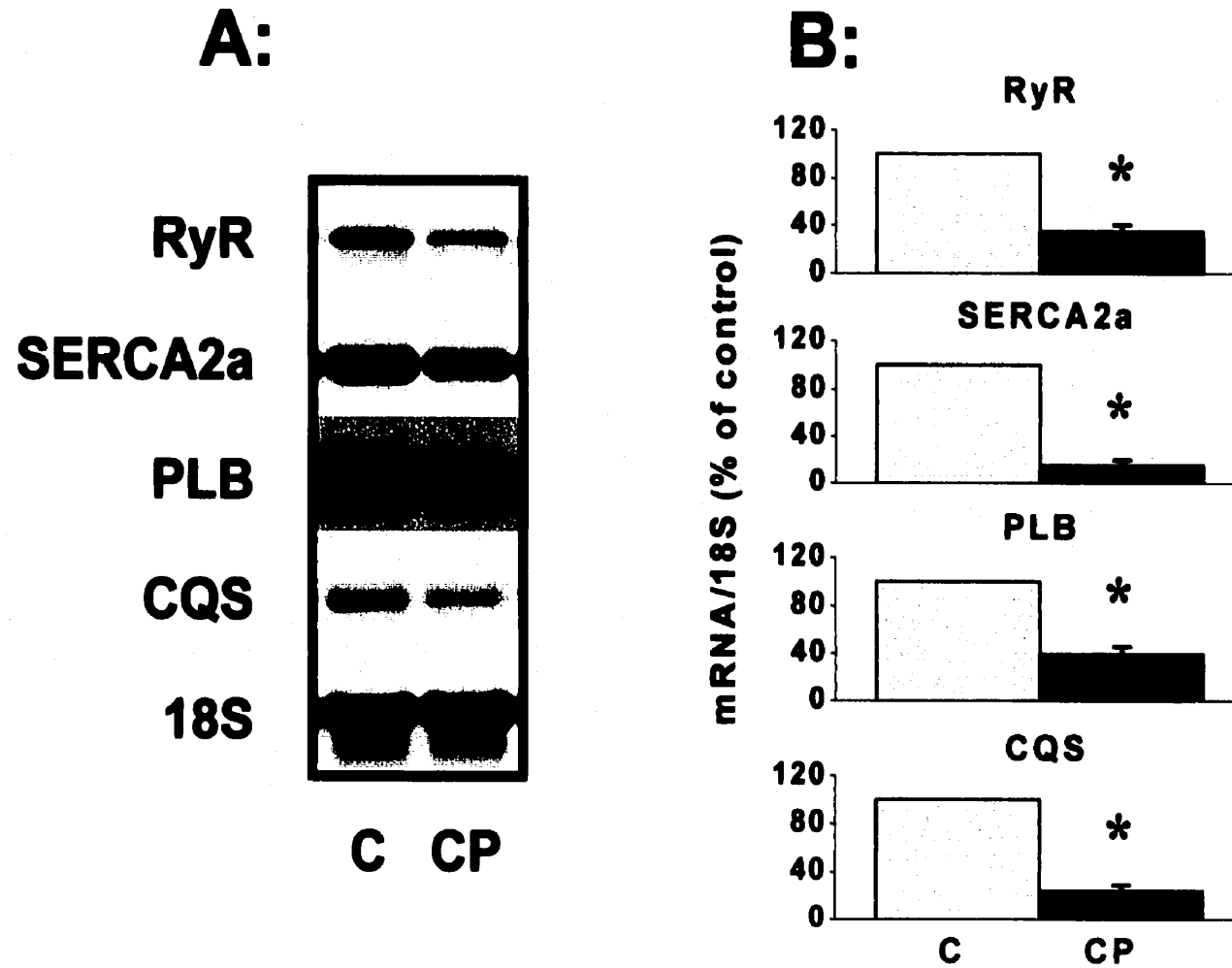
**Table 13: Effect of Ca<sup>2+</sup>-paradox on SR function.**

<b>Group</b>	<b>Ca<sup>2+</sup>-uptake (nmol <sup>45</sup>Ca/mg/min)</b>	<b>Ca<sup>2+</sup>-release (nmol <sup>45</sup>Ca/mg/15 sec)</b>
<b>Control</b>	43.2±5.9	10.8±1.2
<b>Ca<sup>2+</sup>-depletion/repletion</b>	6.8±0.8*	0.4±0.1*

Values are mean±SE of 6 separate experiments. \*P < 0.05 vs. control.



**Figure 21:** Effect of Ca<sup>2+</sup>-paradox on SR protein levels. Panel A shows the autoradiogram and Panel B depicts the analysis of the SR gene expression in control (C), Ca<sup>2+</sup>-paradox (CP). Values are mean  $\pm$ SE of 6 separate experiments. \* P<0.05 vs. C.



**Figure 22:** Effect of Ca<sup>2+</sup>-paradox on SR mRNA expression. Panel A shows the autoradiogram and Panel B depicts the analysis of the SR mRNA expression in control (C), Ca<sup>2+</sup>-paradox (CP). Values are mean  $\pm$ SE of 6 separate experiments. \* P<0.05 vs. C.

## **6. Effect of ischemic-preconditioning on ischemic-reperfused and Ca<sup>2+</sup>-paradox hearts**

### **a. Alterations in cardiac performance and SR function**

IR and CP depressed LVDP, +dP/dt and -dP/dt by 70 to 94%, and increased the LVEDP by 8 to 12 fold in the non-preconditioned hearts (Table 14). IP improved LVDP by 67% in the IR hearts and by 27% in the CP hearts whereas LVEDP was higher than the control level by 4.6 fold in the IR hearts and 5 fold in the CP hearts. IP also improved the recovery of +dP/dt and -dP/dt in both experimental groups. According to previous studies conducted in our laboratory, Ca<sup>2+</sup>-uptake and release activities of IR and CP hearts were significantly depressed when compared to control values (130, 306). These activities were partially recovered by the IP manoeuvres (130, 306).

### **b. Alterations in SR protein levels and mRNA expression**

Reports from our laboratory (130, 306) have shown that the protein levels of IR and CP hearts for RyR, SERCA2a and PLB were markedly depressed as compared to control values. The protein levels were completely or partially protected when the IR and CP hearts were preceded by the IP manoeuvres, respectively.

At the level of SR gene expression, 3 cycles of IP (5'I/5'R) decreased (P<0.05) the mRNA levels for RyR (by 33%), SERCA2a (by 21%), PLB (by 23%)

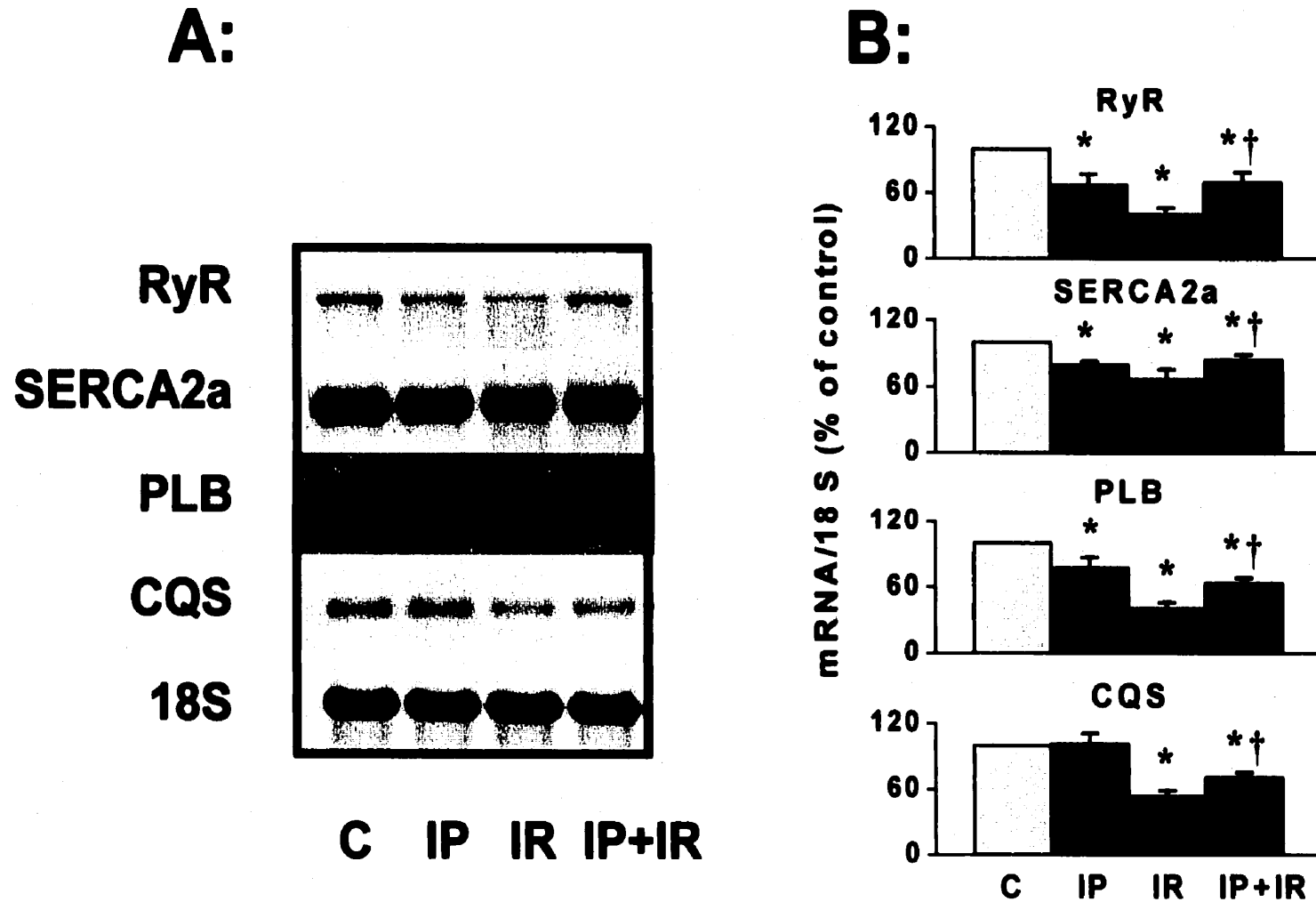
**Table 14: Effect of ischemic-preconditioning on cardiac performance of ischemic-reperfused and  $Ca^{2+}$ -paradox hearts.**

Group	LVEDP	LVEDP	+dP/dt	-dP/dt
	mmHg		mmHg/sec	
Control	84.9±2.0	8.4±0.5	2809±84	1647±73
<b>Ischemia-reperfusion</b>				
IR	21.6±1.9*	102±2.8*	665±35*	498±47*
IP+IR	57.0±2.8*†	39±2.7*†	1985±55*†	1099±51*†
<b><math>Ca^{2+}</math>-paradox</b>				
CP	5.2±1.0*	67±5.7*	375±42*	301±25*
IP+CP	22.4±5.8*†	42±3.2*†	515±24*†	434±20*†

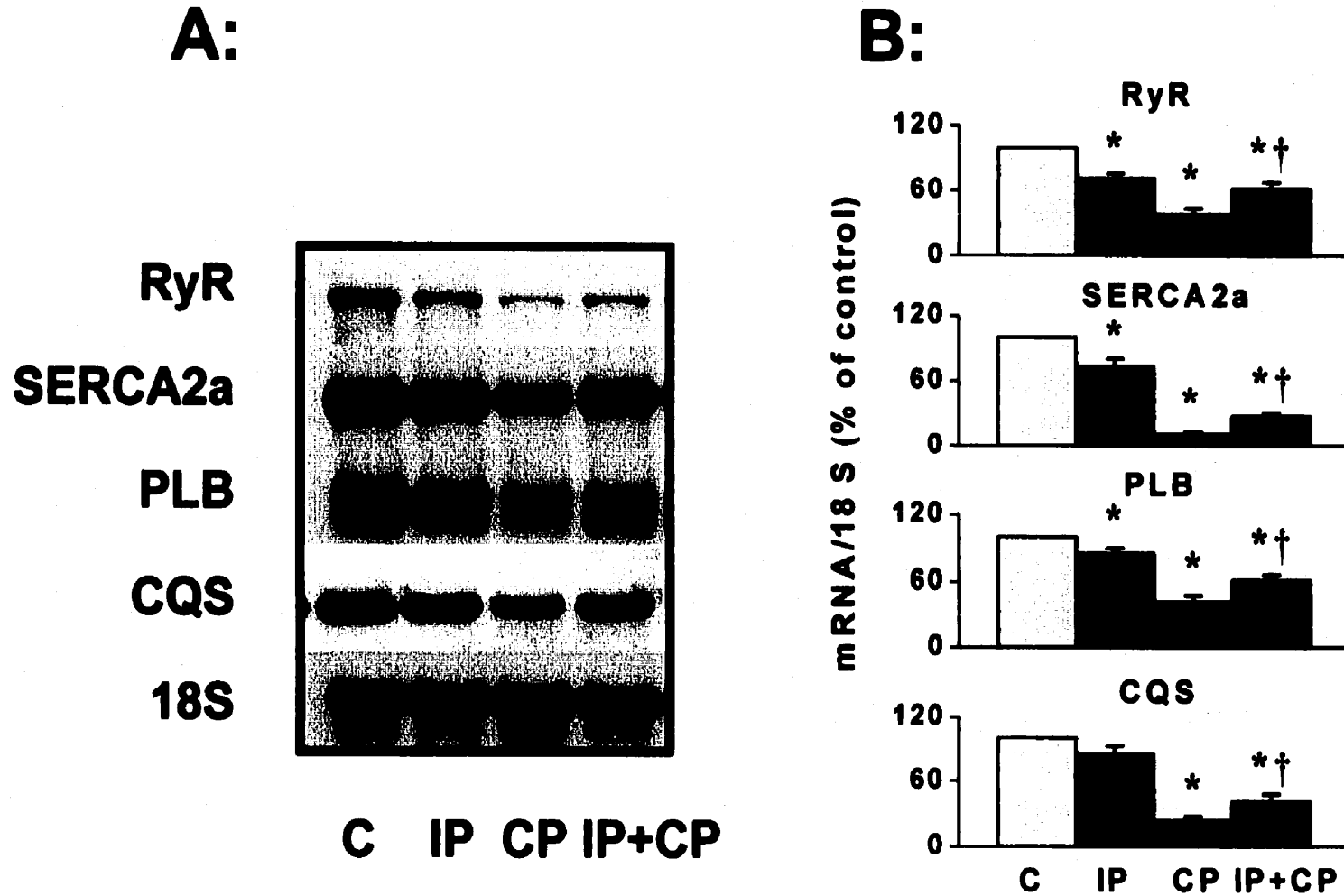
Values are mean±SE of 6 separate experiments. IR, ischemia-reperfusion; IP, ischemic-preconditioning; CP,  $Ca^{2+}$ -paradox. \*P < 0.05 vs. control, † vs. IR or CP.



but had no effect on mRNA levels for CQS (**Figure 23, Panels A and B**). In comparison to the control group, IR decreased mRNA levels for RyR, SERCA2a, PLB and CQS by 60%, 34%, 50% and 46% in the non-preconditioned hearts, respectively. These alterations in SR gene expression were significantly protected by 70% for RyR, 84% for SERCA2a, 62% for PLB and 71% for CQS when IR hearts were preconditioned. Some 30 min ischemic hearts were reperfused for 60 min but the results with respect to changes in SR gene expression were similar to those observed in ischemic hearts reperfused for 30 min. In the CP experiments, 3 cycles of IP (3'I/3'R) significantly decreased the expression of RyR (by 29%), SERCA2a (by 27%) and PLB (by 15%), but not that of CQS (**Figure 24, Panels A and B**). Hearts subjected to CP (5 min  $\text{Ca}^{2+}$ -free and 30 min reperfusion) showed a marked depression in mRNA levels for RyR (by 62%), SERCA2a (by 89%), PLB (by 59%) and CQS (by 76%) as compared to control hearts. IP cycles significantly protected the transcript levels of SR genes in the CP hearts by 61% for RyR, 28% for SERCA2a, 61% for PLB and 41% for CQS.



**Figure 23:** Effect of ischemic-preconditioning on SR mRNA expression in ischemic-reperfused hearts. Panel A shows the autoradiogram and Panel B depicts the analysis of the SR mRNA expression in control (C), ischemic preconditioning (IP), ischemia-reperfusion (IR) and IP plus IR. Values are mean  $\pm$ SE of 6 separate experiments. \* $P < 0.05$  vs. C, † vs. IR.



**Figure 24:** Effect of ischemic-preconditioning on SR mRNA expression in  $\text{Ca}^{2+}$ -paradox hearts. Panel A shows the autoradiogram and Panel B depicts the analysis of the SR mRNA expression in control (C), ischemic preconditioning (IP),  $\text{Ca}^{2+}$ -paradox (CP) and IP plus CP. Values are mean  $\pm$ SE of 6 separate experiments. \* $P < 0.05$  vs. C, † vs. CP.

## V. DISCUSSION

### 1. Ischemia-reperfusion injury

Although it is well established that IR is accompanied by cardiac dysfunction, the underlying mechanisms involved are not fully understood. In agreement with previous reports we have shown that cardiac performance and SR  $\text{Ca}^{2+}$ -uptake and release activities are depressed in hearts subjected to ischemia and subsequent reperfusion (127, 128, 130, 307, 308). It is suggested that depressed immunoreactive RyR and SERCA2a proteins in the ischemic and IR hearts may be a general underlying mechanism contributing to SR dysfunction. As activation of calpain (309) that may degrade high molecular weight proteins such as RyR (211) has been reported to occur in ischemia and reperfusion we felt that the current results were of particular interest (209, 309). We suggest that relatively rapid proteolytic degradation may account for the decrease in protein content observed in our model. We also observed that the extent of decreased protein content of RyR, SERCA2a and PLB genes were unique in ischemic vs. IR hearts. Although the exact reasons for such variation are not clear, it is postulated that the SR proteins may have different sensitivities to cellular injury suggesting that these proteins are differentially regulated. Therefore, studying the rate of protein synthesis and degradation of SR proteins under these experimental conditions may address this issue. In view of the critical role of phosphorylation in regulating the SR function, we have reported that depressed cardiac function in both the ischemic and reperfused heart was accompanied by depressed endogenous CaMK-mediated

phosphorylation of the SR proteins (129). This decrease was accompanied by a concomitant drop in the endogenous CaMK activity (129, 130). Thus, it appears that both SR  $\text{Ca}^{2+}$ -cycling proteins and regulatory mechanisms of SR function are adversely affected in IR-induced injury.

Ischemic injury has been attributed to several factors such as depressed levels of ATP, acidosis, alterations in the osmotic control, glucose deprivation and hypoxia (310-312). Furthermore, the occurrence of oxidative stress, increase in  $[\text{Ca}^{2+}]_i$  and the activation of  $\beta$ -adrenergic system are also considered to be important factors leading to reperfusion injury (86, 249). In an attempt to understand the contribution of some of these factors in IR injury, we investigated the effect of hypoxia and glucose deprivation (i.e. modeling ischemia) and  $\text{Ca}^{2+}$ -overload and oxidative stress (i.e. modeling reperfusion) on IR-induced abnormalities at the level of cardiac performance and SR function. The long-term effect of these factors was determined by investigating the alterations at the level of SR mRNA expression.

## **2. Putative mechanisms underlying cardiac dysfunction due to ischemia-reperfusion injury**

### **a. Hypoxia and /or glucose deprivation**

In agreement with previous reports (299, 313) cardiac performance was depressed in hearts deprived of oxygen and/or glucose. These studies (299, 313) have also shown a decrease in SR  $\text{Ca}^{2+}$ -uptake during the deprivation period. The

omission of glucose from the hypoxic medium was reported to accelerate the deleterious effect of hypoxia on cardiac performance and SR  $\text{Ca}^{2+}$ -uptake (299). These defects were attributed to an insufficiency in energy generation (313) taking into consideration that no changes in membrane lipid composition were observed in the hypoxic and substrate-deprived hearts. In the present study, reperfusion with normal perfusion medium after hypoxia or glucose deprivation resulted in a complete or partial recovery of cardiac performance, respectively; however, a complete recovery of SR  $\text{Ca}^{2+}$ -transport activities was evident. A combination of hypoxia and glucose deprivation was necessary and sufficient to induce abnormalities which were similar to those observed in IR hearts i.e. cardiac dysfunction as well as depressed SR  $\text{Ca}^{2+}$ -uptake and release activities. Although the hypoxic glucose deprived-reperfused hearts did not show any change in the SR protein levels, depressed SR function may be attributed to the alterations in the regulatory mechanisms i.e. decreased CaMK-mediated phosphorylation due to depressed CaMK activity (314).

It can be argued that the defects observed in these hearts may be attributed to other factors such as acidosis or alterations in osmotic control. However, it is important to mention that this may not be applicable under our experimental conditions since the accumulation of metabolic end-products and associated acidosis (315) were prevented by maintaining the coronary flow during the hypoxic and/or glucose deprivation period. Nonetheless, further studies are required to rule out the effect of these factors as well as of alterations in ATP

generation and utilization during the ischemic phase. From this study, it is clear that the absence of both oxygen and glucose, but not either alone, is necessary for the occurrence of reperfusion-injury. An explanation for this phenomenon may be a significant increase in  $[Ca^{2+}]_i$  with a marked elevation in ROS which is reported in isolated adult cardiomyocytes exposed to hypoxia and glucose deprivation (316). Although the reasons underlying the role of oxygen-lack and glucose-lack are not yet investigated, it is clear that  $Ca^{2+}$ -overload and oxidative stress (143, 316, 317) are putative mechanisms responsible for the changes observed in both hypoxia glucose deprivation-reperfused and IR hearts.

#### **b. Oxidative stress**

The role of oxidative stress in IR injury has been shown by direct measurement of ROS levels using different techniques (157-159). Electron leakage from the mitochondrial respiratory chain (137) and auto-oxidation of catecholamines (155) are considered major pathways for the increase in ROS levels. It has been reported that the generation of ROS, during reperfusion after an episode of ischemia, leads to depressed myocardial function (127, 158) both in clinical as well as experimental disease conditions (86, 140, 310). In our study we have demonstrated the role of oxidative stress in IR-induced injury by perfusing the hearts with X plus XO (a superoxide radical generating system) and  $H_2O_2$  which was accompanied by depressed SR  $Ca^{2+}$ -uptake,  $Ca^{2+}$ -release and decreased SR protein levels. The decreasing trend was similar to that observed in IR hearts.

The role of ROS in IR injury was further supported by the cardioprotection provided by antioxidant treatment (discussed later).

**c.  $\text{Ca}^{2+}$ -overload**

Several factors have been implicated in mediating  $\text{Ca}^{2+}$ -overload in ischemic heart disease, some of which are: the activation of sympathetic nervous system and renin-angiotensin system, activation of neutrophils and oxidative stress (137, 249). To study the effect of intracellular  $\text{Ca}^{2+}$ -overload we employed the  $\text{Ca}^{2+}$ -depletion/repletion model ( $\text{Ca}^{2+}$ -paradox) that is known to simulate this condition (318). In agreement with previous reports (306, 319, 320), the impairment of cardiac performance observed in these hearts was consistent with depressed SR  $\text{Ca}^{2+}$ -uptake and release activities. The decrease in the SR function is suggested to be due to depressed SR protein levels and decreased CaMK activity as well as depressed CaMK-mediated phosphorylation of the  $\text{Ca}^{2+}$ -cycling proteins (321). It should be pointed out that the  $\text{Ca}^{2+}$ -paradox model represents a more severe condition than the IR or hypoxia glucose deprivation-reperfusion models, thus the severity of the damage induced by  $\text{Ca}^{2+}$ -depletion/repletion may explain the marked decrease in protein content and cardiac performance observed in the CP hearts.

In view of the above discussion it is clear that hypoxia and glucose deprivation are important ischemic factors that contribute to the reperfusion injury. Moreover, the oxidative stress and the increase in  $[\text{Ca}^{2+}]_i$  that occur during



reperfusion or reoxygenation may be partially responsible for IR injury. These factors may induce their deleterious effect by the proteolytic degradation of the SR proteins or impairment of the activities of SR  $\text{Ca}^{2+}$ -cycling proteins or protein kinases responsible for regulation of the SR function. Nevertheless, the role of lipid peroxidation and decreased levels of ATP cannot be ruled out as contributing factors in mediating the acute (short-term) changes observed in the IR hearts.

### **3. Changes in cardiac mRNA expression**

Although the precise mechanisms by which IR may induce long-term cardiac dysfunction are not clear, the contribution of changes in the SR gene expression cannot be ignored. Our results showed that 30 min of global ischemia significantly reduced the mRNA abundance of RyR, SERCA2a and CQS. Reperfusion for 1 h after 30 min of ischemia resulted in further decrease in the transcript levels of RyR, SERCA2a and CQS from the ischemic level. In addition, there was a reduction in the levels of PLB mRNA that was not observed during the ischemic phase. Therefore, it is possible that different genes have different sensitivities to oxidative stress; the precise mechanisms for this sensitivity are unknown.

In an attempt to understand the possible contribution of ischemic factors to the genetic changes observed in the reperfused hearts, we examined the effects of hypoxia and/or glucose deprivation on the SR mRNA expression. Perfusion of the hypoxic or glucose-deprived hearts with normal oxygenated K-H medium caused no changes in the transcript levels of the SR genes. When hearts were reperfused

after a period of hypoxia and glucose deprivation, a significant decrease in the transcript levels of SR genes was seen. In view of similar alterations in SR mRNA expression observed in the IR hearts, these changes indicate that both glucose-lack and oxygen-lack during the ischemic phase are important for the occurrence of reperfusion-induced injury at the level of the SR mRNA levels. As previously described, these changes may not be attributed to acidosis or abnormalities in the cellular osmotic balance. Nevertheless, further investigation is needed to establish or rule out the role of depression in ATP levels in mediating the changes in SR gene expression.

Depressed mRNA levels of SR genes in IR or hypoxia glucose deprived-reperfused hearts can be explained by two mechanisms, namely the development of oxidative stress and the occurrence of intracellular  $\text{Ca}^{2+}$ -overload (158, 316). There is ample evidence in literature suggesting multiple mechanisms by which oxidative stress may contribute to the alterations in mRNA levels. These include fragmentation of nucleosomal DNA in myocardial IR and alterations in nucleocytoplasmic transport activity (322-324). Exposure of hearts to X plus XO and  $\text{H}_2\text{O}_2$  showed a similar depression in mRNA levels of all the SR proteins as that observed in IR hearts. These results as well as the improvement in SR gene expression due to SOD plus CAT treatment (discussed below) confirm the role of oxidative stress in the observed changes.

$\text{Ca}^{2+}$ -overload has been reported to occur in IR (143) and hypoxia-reoxygenated hearts deprived of glucose (317). Our study shows that  $\text{Ca}^{2+}$ -

depletion/repletion, which is known to cause a massive increase in  $[Ca^{2+}]_i$  (318), induces a dramatic decrease in the expression of RyR, SERCA2a, PLB and CQS. However, on the basis of the information at hand, it is difficult to determine the cause-effect relationship between intracellular  $Ca^{2+}$ -overload and oxidative stress as well as their direct modulatory effect on SR gene expression in the IR hearts.

As both ischemic and IR hearts showed a decrease in the SR mRNA and protein levels, depressed SR activities and cardiac function may be due to decreased SR protein levels as a result of reduced mRNA levels. Nevertheless, this is not the case in our model for several reasons: (a) cardiac function due to IR ceases immediately after 1 min of ischemia whereas the expression of some of the SR genes (i.e. PLB – a regulatory phosphoprotein) does not change even after 30 min of ischemia, suggesting that the functional changes are independent of the alterations at the gene level, (b) treatment with SOD plus CAT did not protect the SR protein content but its beneficial effect was evident at the level of cardiac function and gene expression, (c) the time course of the experimental model is short and this period may not be sufficient for *de novo* protein synthesis and (d) the growing body of evidence shows that changes in protein levels are not necessarily a reflection of changes in the mRNA levels. Therefore, it is suggested that under our experimental conditions the protein content and mRNA expression are regulated independently by different mechanisms. As our data represent the steady-state level, the changes in transcript levels can be attributed to either decreased rate of transcription and/or decreased mRNA stability. Nevertheless,

alterations in mRNA levels in the stunned myocardium as well as during differentiation and cardiac myogenesis have been indicated to be primarily due to changes in the transcription rate (325-327).

At this point, it is important to mention the possible role of thyroid hormone (280) and Ang-II (98) in the modulation of SR gene expression. As the isolated rat heart model is deprived of the effect of circulating thyroid hormone and treatment with captopril or losartan did not exert a beneficial effect on the expression levels of SR genes in the isolated rat heart (232), it is suggested that both thyroid hormone or local Ang-II cannot be factors mediating the IR-induced changes in SR gene expression.

In view of the importance of cardiac mRNA expression in mediating the function of cardiac proteins, the observed changes in mRNA levels for SR proteins due to IR may reflect a delay (long-term) in the recovery of SR function and cardiac performance in the ischemic heart subsequent to establishing reflow. Therefore, further studies are required to address this hypothesis such as a time course follow up of changes in protein and gene expression in an *in vivo* model. Moreover, further study of the transcription regulatory factors such as the *cis*- and *trans*-acting factors that modulate the SR gene expression is required.

### **3. Potential therapeutic strategies**

#### **a. Antioxidants**

In agreement with a previous report (127), we have shown functional recovery in IR hearts treated with a combination of antioxidants. Although treatment with SOD plus CAT improved cardiac performance and SR function, it failed to protect the protein levels of RyR, SERCA2a and PLB. In this regard, Davies and Goldberg (328) reported that different antioxidants protect the erythrocytes against lipid peroxidation but do not prevent proteolysis when exposed to oxidative stress. Because oxyradicals are known to promote lipid peroxidation in SR membranes (329), it is possible that the beneficial effect of SOD plus CAT treatment in the IR hearts may be due to the prevention of SR lipid peroxidation. Moreover, it is likely that ROS may induce their deleterious effect on the SR function by modifying the sulfhydryl groups of the SR proteins such as those of the SR Ca<sup>2+</sup>-pump (330) and the Ca<sup>2+</sup>-release channel (259). Therefore, in view of the restoration of SR function with SOD plus CAT treatment and the pivotal role of Ca<sup>2+</sup>-uptake and release in cardiac contraction and relaxation, the recovery of myocardial function observed in the IR treated hearts may partly be due to improved SR Ca<sup>2+</sup>-handling. Furthermore, we have also demonstrated that the protection of SR function in SOD plus CAT treated IR hearts may be attributed to significant improvement in SR protein phosphorylation (129).

As treatment with SOD plus CAT improved the SR gene expression of IR hearts, it is suggested that antioxidant treatment may promote the long-term recovery of the IR heart. These observations again corroborate the possible role of oxidative stress as a contributing factor in IR-induced injury. The partial recovery

in mRNA levels observed in IR hearts treated with SOD plus CAT indicates that mechanisms other than oxidative stress are involved in the changes observed in the IR hearts. In view of the above discussion, it may be suggested that antioxidant treatment may be beneficial for the short- and long-term recovery of the IR heart (327).

**b.  $\beta$ -adrenoceptor blockers**

Increased activity of the sympathetic system (234) as well as 100-1000 fold increase in the release of norepinephrine from the nerve endings within 20-40 min of ischemia (235) have been reported to mediate myocardial cell damage. Treatment with  $\beta$ -AR blockers rendered the heart protected against some of the abnormalities, including a reduction of the infarct size (236), an attenuation of arrhythmias (133), an improvement in the ventricular function and an overall decrease in mortality (237) which are commonly observed in ischemic heart disease.  $\beta$ -AR blockers have also been used as an alternative to cardioplegic arrest during coronary artery surgery to reduce ischemic damage (331). Although the exact mechanisms for the cardioprotective action of these agents are not yet fully understood,  $\beta$ -AR blockers are considered to exert beneficial effects on the ischemic heart by lowering myocardial oxygen consumption as a consequence of reduced contractility and heart rate, increasing oxygen delivery due to coronary artery dilation (238, 239).

In this study, treatment of IR hearts with atenolol, a  $\beta_1$ -specific blocker, or propranolol, a non-specific  $\beta$ -blocker, significantly improved cardiac performance and SR function. The recovery of cardiac performance attained with propranolol is in agreement with other studies (332, 333). Although previous studies reported no mechanical recovery with atenolol (332, 334), our results indicate a significant recovery with atenolol. This may be due to differences in experimental models, species and concentrations of the drug used. Nonetheless, the beneficial effects of  $\beta$ -AR blockers in IR hearts are well documented (234, 236, 335). Our results show that the depression in  $\text{Ca}^{2+}$ -uptake and release activities in IR hearts were recovered upon administration of  $\beta$ -AR blockers and this may account for the improvement in cardiac performance. The protection provided can be attributed to several factors: a) the treatment ameliorated the depression in the protein levels of the SR  $\text{Ca}^{2+}$ -cycling proteins that was observed in the IR hearts, this beneficial effect may be due to the action of  $\beta$ -AR blockers on proteolysis and cannot be accounted for *de novo* protein synthesis, b) we have also shown an improvement in the CaMK and PKA phosphorylation of the SR proteins (336) due to  $\beta$ -AR blockade, therefore the protection of SR regulatory mechanisms cannot be ruled out, and c) the  $\beta$ -blocking properties of the drugs because the concentrations of both atenolol and propranolol prevented the positive inotropic effect of isoproterenol completely.

As an excessive amount of catecholamines released during IR may alter  $\text{Ca}^{2+}$ -transport mechanisms resulting in intracellular  $\text{Ca}^{2+}$ -overload (128),  $\beta$ -AR blockade may attenuate these deleterious effects and render cardioprotection. Furthermore, the improvement in cardiac performance, SR function as well as protection at the level of SR proteins in hearts treated with propranolol was significantly higher than in the hearts treated with atenolol. This difference may be attributed to the properties, such as higher lipophilicity, membrane-stabilizing activity (241), antiperoxidative activity (242), and antiradical effect (337) of propranolol in comparison with those of atenolol. It is important to mention that propranolol also inhibits cardiac phospholipase A (PLA) (338). The activation of  $\text{PLA}_2$  has been reported to occur as early as 30 min of coronary ligation which significantly contribute to the degradation of membrane phospholipids and the production of large amounts of lysophospholipids (339). Furthermore, propranolol, unlike atenolol, has been reported to prevent the ischemic-induced release of norepinephrine from the sympathetic nerve endings in the heart (297, 298) and thus other actions of  $\beta$ -AR blockers cannot be excluded. Accordingly, it is suggested that  $\beta$ -AR blockade as well as the ancillary properties of propranolol may contribute towards its cardioprotective effects in IR hearts.

In a model of congestive heart failure, sympathetic stimulation was found to be partially responsible for the reduction of SR  $\text{Ca}^{2+}$ -ATPase mRNA (97). Our results show that IR caused a decrease in the mRNA abundance of RyR,



SERCA2a, PLB and CQS. The protective effect of propranolol on IR induced changes in the levels of mRNA for SR proteins are consistent with the antioxidant and  $\beta$ -blocking properties of propranolol. It can be argued that the improvement in SR mRNA expression is related to improved SR protein levels. Nevertheless, as discussed in the previous section, this relationship is of questionable significance. However, atenolol which has no antioxidant property was also found to protect changes in SR gene expression due to IR. These results indicate that the recovery of mRNA expression of the SR proteins by  $\beta$ -AR blockers may be independent of an antioxidant property. Thus, the observed changes in SR gene expression due to IR in control and drug-treated hearts cannot account for the depression in SR  $\text{Ca}^{2+}$ -transport activities and cardiac performance under the experimental conditions employed in our study.

In conclusion,  $\beta$ -AR blockers may provide short-term cardioprotection by improving SR function and regulation and provide long-term protection by preventing the changes in the SR gene expression in IR hearts. To the best of our knowledge, this is the first study to report the beneficial effects of atenolol and propranolol in terms of improved cardiac performance of the ischemic heart, which may occur at the level of SR function as well as SR gene expression. Since the protection by  $\beta$ -AR antagonists with respect to IR-induced changes in SR function and contractile performance were partial in nature, this study does not exclude the participation of other factors in the genesis of IR injury. In addition,

the beneficial effect of  $\beta$ -blockade in attenuating the IR-induced changes in mRNA levels for SR proteins can also be seen to support the view regarding cardiac gene expression as a molecular site for the cardioprotective action of  $\beta$ -blocking agents.

### **c. Ischemic-preconditioning**

IP has been shown to be an effective intervention for attenuating the IR-induced changes in the heart since it had a remarkable effect on the IR-induced alterations in infarct size, arrhythmias, cardiac performance, SR function and protein content (130, 250, 251, 253, 254, 340). Moreover, it is documented that IP reduces the rise in intracellular acidosis, intracellular  $\text{Na}^+$  and  $\text{Ca}^{2+}$ -concentrations due to IR (252), in addition to inhibiting glycolysis and maintaining glucose oxidation (341). In agreement with our previous reports, IP prevented the changes in cardiac performance of IR and CP hearts (130, 251). The improvement in the protein levels, the CaMK-mediated phosphorylation of the SR proteins as well as the CaMK activity (130, 251) in these models may account for the improvement in cardiac performance.

At the level of SR mRNA expression, a decrease in the transcript levels for SR proteins such as RyR, SERCA2a, and PLB, but not CQS, were observed in the IP cycles. These results suggest that repetitive cycles of IP decrease the transcript level of cardiac gene and this effect is not of a generalized nature, indicating that these genes are differentially regulated under the same conditions. Studies

conducted by Schaper and co-workers (325, 342) showed an increase in the transcript levels for SERCA2a, PLB and CQS due to one cycle of 10 min coronary occlusion and 90 min of reperfusion (325) whereas two cycles of 10 min occlusion followed by reperfusion for 60 min under *in vivo* conditions showed a tendency towards a decrease in the mRNA levels. In this particular study (325), the absence of changes in the mRNA levels of some cardiac genes was suggested to indicate that IR did not damage the protein and therefore there was no change in the transcript levels (342).

Although IP cycles caused a significant decrease in mRNA levels for the SR genes, these short episodes of IP were able to precondition the SR genes against the deleterious effect of IR- or CP-induced injury. The protection provided by three cycles of IP (3 or 5 min duration) was not surprising since IP cycles longer than 1 min were shown essential to induce functional protection (255). At this point it is important to mention that the contractile recovery observed in IR hearts due to IP would be mainly related to the preserved protein content of subcellular organelles (130, 306) and not linked to the status of the mRNA expression (343). On the other hand, it is likely that IP may rescue the SR protein levels from degradation by  $\text{Ca}^{2+}$ -dependent proteases that are activated during IR (210, 309). Although this possibility needs to be proven, it may be valid since one episode of IP is sufficient to reduce the elevation in  $[\text{Ca}^{2+}]_i$  in subsequent ischemic episodes (344). Thus, it appears that different mechanisms may be involved in improving SR function in the IR hearts by IP.

In conclusion, this study indicates that although IP may appear deleterious, this phenomenon seems to trigger yet unknown mechanisms that mediate protection of cardiac SR gene expression against cellular injury. The results of this study also provide novel insights into the mechanisms involved in the modulation of the SR mRNA expression and add to the existing knowledge in the area of ischemic preconditioning.

#### **5. Significance of the study in relation to ischemic heart disease**

Experimental and clinical studies have extensively investigated the defects in cardiac performance and SR function under different pathological conditions. These defects have been attributed to abnormalities in  $\text{Ca}^{2+}$ -transport, alterations in the SR protein levels and mRNA expression of the SR genes. Nevertheless, these studies have been conducted in different cardiac diseases especially at a terminal stage of heart failure whereas no report was available whether such changes occur in IR hearts. Moreover, the pathogenic factors contributing to cardiac dysfunction and alterations in protein content as well as mRNA expression levels were not investigated. Our study is the first to report the significant role of hypoxia and glucose deprivation in the reperfusion-induced injury. It is possible that the absence of both oxygen and glucose contribute to oxidative stress and the increase in  $[\text{Ca}^{2+}]_i$  which may then induce IR injury.

Thus, our study provides direct and indirect evidence to support the role of oxidative stress and intracellular  $\text{Ca}^{2+}$ -overload as possible mechanisms

contributing to the acute (short-term) and chronic (long-term) SR abnormalities in the IR hearts. We also directly showed that the depressed SR function is not necessarily related to abnormalities in the content of the  $\text{Ca}^{2+}$ -cycling proteins. This was evident in the hypoxic glucose deprived-reperfused hearts where the depressed SR function was not associated with any alterations in the SR protein levels. Moreover, changes in protein content may not emulate the alterations at the transcript level. Both the differential changes in the SR protein levels and mRNA expression between different experimental groups indicate that different proteins and their transcripts are regulated independently. These results can be seen to open the way for investigations regarding various factors contributing to the activation or suppression of these genes and hence extend our understanding of new targets for gene therapy.

## VI. CONCLUSIONS

In summary, this study provides direct and indirect evidence for the role of oxidative stress,  $\text{Ca}^{2+}$ -overload, hypoxia and glucose deprivation in depressed SR gene expression and SR dysfunction in the IR heart. These factors may contribute to a short-term (acute) or long-term (chronic) dysfunction in the myocardium. Short-term changes, reflected by depressed SR function and hence defects in cardiac performance, may partially be due to alterations at the level of SR protein content. On the other hand, changes in the SR transcript levels may be the underlying mechanism for the long-term cardiac dysfunction due to IR injury. Treatment with a potent oxyradical scavenger system such as SOD plus CAT or  $\beta$ -AR blockers provide short and long-term cardioprotection. The short-term beneficial effects may be related to protecting SR function and protein content. On the other hand, the long-term beneficial effect may be a consequence of preventing changes in SR protein gene expression. Therefore, our results suggest that the administration of antioxidants or  $\beta$ -AR blockers during surgical interventions would go a long way in the clinical management of cardiovascular diseases. Our study is the first to demonstrate that a combination of hypoxia and substrate deprivation, unlike hypoxia or substrate-lack alone, play an important role in modulating the genetic machinery in IR hearts. The alterations observed in these IR-hearts may be attributed to  $\text{Ca}^{2+}$ -overload and/or oxidative stress that occur during the reperfusion phase.

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