ROLE OF ANGIOTENSIN IN SARCOPLASMIC RETICULUM REMODELING IN HEART FAILURE DUE TO MYCARDIAL INFARCTION

by

Xiaobing Guo

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Role of Angiotensin in Sarcoplasmic Reticulum Remodeling in Heart Failure due to Myocardial Infarction

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ABSTRACT

Although remodeling of the sarcoplasmic reticulum (SR) membrane with respect to Ca^{2+}-regulating proteins has been shown to occur in congestive heart failure (CHF), the mechanisms are poorly understood. In this study, we tested the hypothesis that the beneficial effects of enalapril (angiotensin converting enzyme inhibitor) and losartan (angiotensin II receptor antagonist) on heart function in CHF induced by myocardial infarction (MI) are associated with improvement of SR Ca^{2+}-regulating protein function. Three weeks after coronary occlusion, Sprague-Dawley rats were given enalapril (10 mg/kg/day) or losartan (20 mg/kg/day) orally for 4 weeks. Ligation of the left coronary artery in rats for 7 weeks induced the appearance of large Q-waves, scarring of the left ventricular wall, cardiac hypertrophy and CHF, as reflected by lung edema. The left ventricular end diastolic pressure was increased whereas +dP/dt and -dP/dt were depressed without any alterations in the heart rate or left ventricular systolic pressure. Treatment of infarcted animals with enalapril or losartan partially prevented the pathophysiological hemodynamic changes without significantly affecting the scar weight or the magnitude of the Q-wave. Cardiac performance in the sham control animals was not affected by enalapril or losartan treatment. Western blot analysis of SR proteins revealed depressions in Ca^{2+}-release channel, Ca^{2+}-pump ATPase and phospholamban contents without any change in the calsequestrin content in infarcted hearts. The mRNA levels assessed by Northern blot for Ca^{2+}-release channel, Ca^{2+}-pump ATPase and phospholamban, but not calsequestrin, were also decreased in the infarcted hearts. The observed alterations in SR protein content and gene expression in the infarcted animals were partially prevented by therapy with enalapril or losartan. No alterations in SR

protein content and gene expression were seen upon treatment of sham control animals with enalapril or losartan. These results suggest that alterations in SR Ca^{2+}-regulating proteins in congestive heart failure following myocardial infarction may be a consequence of altered gene expression. Furthermore, the beneficial effects of enalapril and losartan in heart failure may be due to the ability of these agents to prevent remodeling of the SR membrane by modification of altered gene expression in the failing heart.

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I. REVIEW OF LITERATURE

Congestive heart failure (CHF) represents an enormous clinical problem and is a symptomatic syndrome in which cardiac output is inadequate to meet the metabolic needs of the human body (1). In the past several decades, two characteristic epidemiologic changes have been observed in the field of CHF: Firstly, the incidence and prevalence of CHF and the resulting morbidity and mortality have dramatically increased whereas the mortality and morbidity from most other cardiovascular diseases have decreased greatly. In the United States of America alone, there are 400,000 new cases of CHF each year and more than 4.7 million people suffer from this condition. In fact, CHF has become a major social and economic burden; the overall cost of managing CHF is estimated to be about $15 billion. Furthermore, the 5 year survival rate of CHF is as low as 25% in men and 38% in woman (2, 3). Secondly, the etiologies of CHF have significantly shifted from hypertension and valvular heart disease to coronary artery disease and diabetes (3). Although etiologies of CHF include ischemia and myocardial infarction (MI), sustained hemodynamic pressure (hypertension) or volume overload, and cardiomyopathies (myocarditis, dilated or hypertrophic cardiomyopathy) (4), the most frequent cause of heart failure in developed countries is ischemic heart disease (3). This review is therefore focused on discussion of the pathophysiology and therapy of congestive heart failure subsequent to MI.

1. Heart Failure Following MI

After MI the cardiac pump failure due to the loss of contractile tissue evokes a series

of responses that act to maintain systemic perfusion (2). The structural remodeling of the left ventricle (LV) after MI is a time-dependent process, which involves complex alterations in ventricular architecture including both the infarcted necrotic heart and noninfarcted viable myocardium. Increased systolic and diastolic wall stress due to inefficient emptying of the LV induces structural alterations in response to the enlarged cardiac volume (5, 6). Although contractile dysfunction may occur acutely in MI, heart failure usually arises more gradually and incrementally following the progressive LV dilatation and remodeling of the viable myocardium. An acute myocardial infarction, particularly with a large and transmural infarct, can produce alterations in the topography of both the infarcted and noninfarcted areas of the ventricle (5, 7). Within hours of MI, necrosis, edema and inflammation are localized to the infarcted area, which are followed by a long-term period of fibroblast proliferation and collagen deposition. This is referred to as scar formation, which is completed within weeks to months depending on species, and for rats the period of scar healing is about 3 weeks (2, 5). At the same time, the infarct expansion or thinning of the area of infarction occurs and this results in LV dilatation (2, 5, 6, 8, 9); it is likely to be associated with complications such as the development of CHF, aneurysm formation and myocardial rupture (5).

As a compensation to maintain the stroke volume after the loss of contractile tissue, ventricular enlargement and hypertrophy of the viable myocytes take place. MI initiates time-dependent secondary changes in the noninfarcted tissue (5, 6, 8-10). While the sarcomeres from the enlarged human heart may be normal in length, the volume increase of the impaired ventricle may be due to a rearrangement of the myofibrils across the wall

(myocyte slippage or a decrease in the number of cells), rather than a simple stretching of sarcomeres (2, 5). Whenever a precarious balance is exceeded, the increased cavity volume with insufficient compensatory hypertrophy and the resulting loading conditions further promote cardiac enlargement and dysfunction (2, 5). These changes after MI are regarded as the acute phase of ventricular remodeling whereas the chronic phase occurs when the stimulus to volume enlargement still exists but the healing process of scar is complete (2).

It is well known that the remodeling process following MI is considerably more complex than that in any other models of pure hemodynamic overload. The process of remodeling is targeted to the heart as an organ and includes all cell types present in the heart: myocyte, interstitial cells and fibroblasts in particular, and the vascular endothelium and the immune cells in some cases (11). In the MI model, hemodynamic factors (increased left ventricular volume, increased systolic and diastolic wall stresses), necrotic factors, inflammation factors and myocardial ischemia exist and mix together (12). The growth of vessels around the infarcted area normalizes the coronary vasodilatory capacity in 35 days (13). In addition, the activation of the sympathetic nervous system and renin-angiotensin system (RAS) cause an enhancement in loading conditions in the failing ventricle and may accelerate the progression of CHF. The release of neurohormones in CHF not only exacerbates the hemodynamic abnormalities but their continuous release also initiates a series of self-reinforcing events which lead to LV dysfunction and CHF (14). It was reported that high concentrations of neurohormones, such as atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in plasma, may affect both the LV dilatation and systolic

dysfunction and play an important role in remodeling of the LV (15). In addition, coronary artery occlusion promotes the accumulation of collagen, which decreases myocardial contractile strength, increases cardiac stiffness and initiates the expansion of the infarcted tissue (16).

Under clinical conditions, the acute and long-term changes in ventricular remodeling after MI are the essential processes affecting ventricular function and survival. In addition to the initial insult during MI, the ventricular remodeling also has an important impact on the ultimate outcome after MI and thus its therapy is important (5, 17). It is also considered that remodeling encompasses the transition from hypertrophy to CHF (18); when the cardiac dilatation and geometry reach a critical point of heart weight (11), the LV remodeling itself may contribute to progression of CHF (4). In terms of cellular alterations during the development of CHF, myocytes and fibroblasts play a key role in remodeling, but cardiomyocytes constitute the majority (76%) of the heart (11) and are more important. At the end-stage of heart failure in patients, remodeling of LV myocardium after MI is commonly associated with interstitial fibrosis in the noninfarcted hypertrophic myocardium which is remote from the scar area (19).

2. MI Rat Model of Heart Failure

The analysis of CHF relies on the use of appropriate and complementary experimental model systems; however, no animal model has all the salient pathophysiological features desired to mimic the clinical human conditions. Nonetheless, the post-MI rat is most

frequently used as an experimentally-induced model of CHF (18, 20-22). The rat model of MI with coronary artery ligation was first documented by Johns and Olsen as cited by Dhalla et al. (1). The procedure for the rat model of MI with coronary artery ligation has improved gradually and has been applied extensively to study CHF with respect to morphological features, metabolic and mechanical adaptations and therapeutic evaluations (1, 2, 22-34). It is now well accepted that left coronary artery ligation induces sustained and severe MI in rats as the necrotic myocardium becomes completely replaced by scar tissue within 3 to 4 weeks (24); cardiac failure begins to occur by 4 weeks after the induction of myocardial infarction (1, 35, 36). CHF can be confirmed by hypertrophy of the viable myocardium, congested lungs and hemodynamic alterations, including reduction of mean arterial blood pressure (MAP), left ventricular systolic pressure (LVSP), left ventricular positive and negative dP/dt in addition to a rise in left ventricular end-diastolic pressure (LVEDP). The defect in cardiac contractile function and loss of responsiveness to sympathetic stimulus, which are the hallmarks of failing human myocardium, are also observed in the MI rats. Rats with small infarcts have systolic dysfunction with reduced contractility and peak pressures, and those with large infarcts also have diastolic dysfunction with increased filling pressure. In some cases the presence of ascites has also been reported (1, 2, 20, 23, 24, 26, 36-39). The depression of LV function depends on the size of scar formation; a scar size of 30-40% (24) or greater (1, 23) of the LV has been reported to induce CHF. In the MI rat model, a large Q-wave (> 1 mV) is considered to have developed acute myocardial infarction which is followed by heart failure (36). Only animals with large Q waves in leads I, aVL and V_5

were utilized by some investigators and those with LVEDP < 16 mm Hg were excluded from the infarcted groups (12).

In the MI model of CHF, the LV not only loses a part of its contractile mass but the remaining myocardium also develops hypertrophy; this viable tissue has been used to study the structure, functional and morphological characteristics of surviving myocardium subsequent to MI (40). The LV volume increased progressively as a time-dependent process that is related to the extent of histological damage. Although the predominant metabolic changes in the viable myocardium occur early during the thinning and expansion of the infarct, both dilation and eccentric hypertrophy of the viable myocardium also commence early and continue long after the MI healing (5). The failing myocardium due to MI develops a lengthwise hypertrophy with series replication of sarcomeres whereas the additional sarcomeric proteins assemble in parallel in the pressure overload model (41); the surviving myocardium is altered leading to the development of CHF (41, 42). There is quantitatively a similar degree of cardiac myocyte hypertrophy, a 31% increase in cell volume compared to most models of pressure-overload (12). A depression in the mechanical function in cardiomyocytes isolated from the failing myocardium 6 weeks after MI has been reported (41) and the same phenomenon was observed in the skinned cardiac fibers as well (43). However, some investigators have reported normal contractile function of cardiomyocytes from the infarcted failing rat heart (44). In the MI rat, the collagen concentration appears to double in the viable free wall of the LV but increases by 27% in the noninfarcted septum (2). Several therapeutic interventions have been tested in the MI rat

model. Recombinant human growth hormone (GH) produced a 13% increase in the ejection fraction (EF) and a 50% increase in cardiac index, while there were no signs of additional hypertrophy (32). However, GH replacement failed to improve LV function in hypophysectomized rats after MI (45). Long-term administration of coenzyme Q_{10} was found to increase the cardiac output (CO) and stroke volume slightly but did not affect the survival rate (46). The main disadvantage of the MI rat model is the high mortality, 40-50% in the first 48 hours (24, 25); however, this experimental model has been shown to be quite reproducible.

There are several related animal models of CHF or cardiac hypertrophy, which have been reported to exhibit ventricular remodeling. For example, MI in dog has been shown to result in elevated LVEDP and decreased stroke volume (47). Rabbits after MI were used to evaluate changes in the sarcoplasmic reticulum (SR) and sarcolemma (SL) function due to heart failure (48). Chronically diabetic animals induced by streptozotocin exhibited significantly depressed contractile force generation, SR Ca^{2+}-transport and Ca^{2+}-stimulated ATPase activities; these alterations were reversed by insulin therapy but not by thyroid hormone (49). Depressed Ca^{2+} mobilization and positive inotropic responses were also observed in this model of contractile dysfunction (50, 51). Heart failure due to aortic stenosis or pressure overload in guinea pigs (52, 53), felines (54), ferrets (55) and rats (56, 57) is induced by banding the descending thoracic aorta. Pressure overload in the rats is commonly induced by suprarenal abdominal aortic coarctation; these rats exhibit increased systolic LV pressure and LV hypertrophy (58, 59), as well as reduced mRNA levels for SR

Ca^{2+}-regulating proteins (29, 60). The genetically hypertensive rat belongs to the pressure overload model (61). Volume-overload in rats is induced by an aortocaval shunt and the cardiac hypertrophy thus induced is considered to produce compensatory changes for maintaining LV function (62). Tachycardia in pigs and dogs has also been shown to result in CHF. The SL L-type Ca^{2+}-channel and SR Ca^{2+}-pump densities were reduced in the failing pig heart; these changes were recovered by a combination therapy with an angiotensin-converting enzyme inhibitor (ACEI), benazeprilat and angiotensin II (Ang II) type I receptor (AT_1) blocker valsartan; neither ACEI nor AT_1 receptor blocker alone had such effects (63). In the tachycardia-induced CHF in dogs, it was shown that both the activity and protein level of SR Ca^{2+}-pump ATPase were reduced (64, 65). The cardiomyopathic Syrian hamster has also been widely used for the study of both hypertrophic (BIO 14.6) (66) and dilated (BIO 5.58) (67, 68) cardiomyopathy.

3. Role of Ca^{2+} in Heart Function

The LV function alterations in CHF may be due to changes in Ca^{2+}-handling, sarcomerogenesis, β-adrenergic signaling and cytoskeleton (4). However, cardiac muscle contraction and relaxation are mainly regulated by the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$), which is controlled by the SR Ca^{2+}-release and Ca^{2+}-uptake processes. SR is a tubular network of membranes which participates in cardiac contraction and relaxation by raising and lowering the cytoplasmic levels of Ca^{2+} (69, 70). Most interventions that affect the strength of cardiac muscle contraction appear to exert this effect by altering $[Ca^{2+}]_i$ and/or

the sensitivity of the contractile apparatus to Ca^{2+} (1, 70-75). Cardiac muscle contraction begins with Ca^{2+}-influx through SL Ca^{2+}-channels and ends with Ca^{2+}-uptake by SR (1, 36, 73). Abnormalities in the intracellular Ca^{2+}-handling are considered to represent the basis of depressed contractility in CHF (1, 75). Alterations in myocyte function are ascribed to changes at three sites in the excitation-contraction coupling (E-C coupling): the SL L-type calcium channel opening, Ca^{2+}-release from the SR and the reaction of myofilaments to Ca^{2+} (1, 41, 73) (Fig. 1).

The resting $[Ca^{2+}]_i$ is low (<200 nmol/L) whereas the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) is high (1 mmol/L) and thus there is more than a 5,000-fold gradient across the SL membrane. Various cation channels and transport proteins are involved in maintaining the low level of $[Ca^{2+}]_i$, and these include the SL Ca^{2+}-pump and Na^+-Ca^{2+} exchanger as well as the ryanodine receptor (RYR) and Ca^{2+}-pump ATPase (SERCA) (1, 73). The E-C coupling is a complex process in cardiomyocytes which is initiated by the membrane electric activity. Extracellular Ca^{2+} enters the cardiomyocyte via specific SL Ca^{2+}-channels (voltage-gated, L-type Ca^{2+}-channel), and triggers Ca^{2+}-release from SR via the RYR. This process is called Ca^{2+}-induced Ca^{2+}-release (CICR) (73, 75-77). A "restricted space" where Ca^{2+}-entry via SL is coupled to RYR and is adjacent to the junctional SR seems to play an important role in the process of E-C coupling (78). The increased cardiac contractility in hypertrophied hearts of the spontaneously hypertensive rat was not associated with any changes in the SL L-type Ca^{2+} current or the SR Ca^{2+}-regulating proteins and thus alterations in the microdomain between L-type Ca^{2+}-channels and RYR were suggested to explain this observation (79).

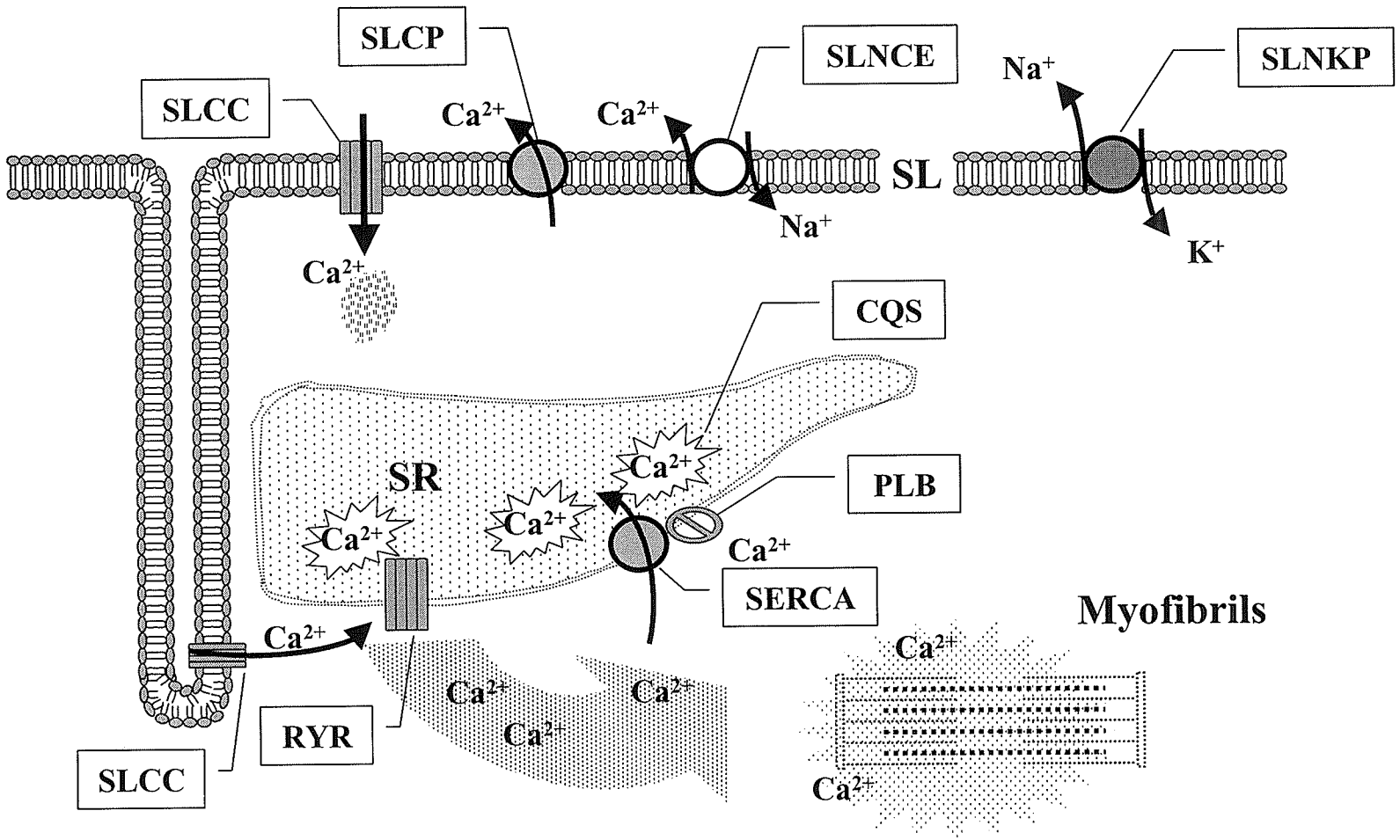

Figure 1. Calcium induced calcium release in cardiomyocyte

SL: sarcolemma; SR: sarcoplasmic reticulum; RYR: ryanodine receptor; SERCA: sarcoplasmic reticulum Ca^{2+} pump; PLB: phospholamban; CQS: calsequestrin; SLCC: sarcolemma Ca^{2+} channel; SLCP: sarcolemma Ca^{2+} pump; SLNKP: sarcolemma Na^{+}-K^{+} pump; SLNCE: sarcolemma Na^{+}-Ca^{2+} exchanger.

While some Ca^{2+} may enter the cell via the reverse of Na^{+}-Ca^{2+} exchanger under certain conditions, Ca^{2+} released from SR is considered to increase the $[Ca^{2+}]_i$ and result in a series of chemical events (cross-bridge formation) which initiate cell shortening and/or the generation of force. In this process of myofilament activation, most of the Ca^{2+} that causes contraction comes from SR (73, 78); however, both endothelin and β-adrenergic agonists have also been shown to increase actomyosin ATPase activity directly (80). In the last step of E-C coupling, Ca^{2+} interacts with troponin C, the regulatory complex of the contractile apparatus, to initiate the contraction (1).

The magnitude of contraction in different experimental models of heart failure is directly dependent upon the $[Ca^{2+}]_i$ in cardiomyocytes (1, 27, 73, 74, 79). In failing hearts due to MI in rats, the diastolic $[Ca^{2+}]_i$ was increased and the systolic peak $[Ca^{2+}]_i$ was decreased while the contractile force development and relaxation of heart were reduced (27, 81, 82). The time course of SR release function in the rabbit model was tested by the caffeine-induced contracture and it was observed that both Ca^{2+}-release and Ca^{2+}-uptake were increased at 8 weeks but were normal at 15 weeks after MI (83). It should be pointed out that not only the cardiac SR function is altered during CHF after MI but the skeletal muscle SR is also affected. There is significant reduction in the skeletal muscle twitch and tetanic forces in addition to significant elevations in the rates of tension increase and decrease in the infarcted animals (84). Accordingly, it has been concluded that both the cardiac and skeletal muscle SR functions are altered following MI.

4. SR Ca^{2+}-Regulating Proteins and Their Function

Due to its ability to take up and release free Ca^{2+}, SR is considered to play a key role in the process of E-C coupling for the regulation of intracellular Ca^{2+} in the heart on a beat-to-beat basis (1, 36, 73). The regulation of Ca^{2+} by SR is carried out by the Ca^{2+}-regulating proteins such as RYR, SERCA, PLB and calsequestrin (CQS), which control the Ca^{2+}-release, -uptake and -storage activities. The RYR is located on the junctional SR and triggers the contraction of cardiac myocytes by releasing Ca^{2+} from the lumen of SR into the cytoplasm. The RYR isolated from the heart and brain is a large protein of 4,969 amino acids with *Mr* of 564,711 dalton and its gene is located on chromosome 1 (85). When exposed to micromolar concentrations of Ca^{2+}, the RYR is opened and thus releases Ca^{2+} from SR into the cytoplasm of the cell (67, 85-88). The RYR has two important pharmacologic characteristics; firstly, it has a high affinity to an alkaloid ryanodine. At low concentrations (< 10 μmol/l) of ryanodine, the Ca^{2+}-release channel is opened in a state of a low-conductance configuration while high concentrations of ryanodine completely block this channel (89, 90). Therefore, low concentrations of ryanodine are used to test the extent and speed of Ca^{2+} release from SR (90). It was also found that the Ca^{2+}-release induced by inositol 1,4,5-triphosphate (IP_3) was reduced in the pressure-overload hypertrophied heart (56). However, the amount of Ca^{2+} released by the SR depends on the size of Ca^{2+} load in SR and on the size and duration of the initial Ca^{2+} trigger from the L-type Ca^{2+}-channel in SL (91).

The SR Ca^{2+}-stimulated ATPase (SERCA) is localized in the cardiac longitudinal SR

and works as a pump in which ATP is hydrolyzed for the transport of Ca^{2+} (29). SERCA is a 100 kD protein and is a major protein in the SR membrane (1). SERCA is distributed all over the tubular SR (1, 92) and its activity is dependent upon both Ca^{2+} and Mg^{2+}. There are three highly homologous genes in the SERCA pump family: SERCA1 (SERCA1a and SERCA1b) is restricted to fast-twitch striated muscle and has a relatively high Ca^{2+} affinity (93) whereas SERCA2 and SERCA3 are expressed most abundantly in the myocardium, large and small intestine, thymus and cerebellum (94, 95). Two isoforms of SERCA2 (SERCA2a and SERCA2b) (96) differ in their C-terminal sequences as a result of alternative splicing (97); SERCA2a in cardiomyocytes plays a key role for Ca^{2+}-uptake into the SR while SERCA2b is expressed in a variety of smooth muscle and non-muscle tissues (95, 98, 99). Two functional copies of the SERCA2 gene are required to maintain normal levels of SERCA2 mRNA, protein and Ca^{2+}-sequestering activity (97). However, it was found that SERCA1a can substitute for SERCA2a functionally and can be regulated by endogenous PLB in the heart in the transgenic mouse model (100). Both ATP and potassium are known to regulate the function of SERCA2 (92, 101).

Not only the rate and extent of cardiac relaxation, but the rate and amplitude of contraction are also controlled by SERCA2. In the failing human heart, the beat duration was found to be longer than that in the normal heart but this difference of contraction disappeared upon treatment of the normal heart with a SERCA inhibitor, thapsigargin. The alteration of contraction in CHF was suggested to be due to decreased SR Ca^{2+} uptake (102) because the contraction is regulated by the amount of Ca^{2+} in SR as well as the Ca^{2+} gradient

between the SR and the cytosol (75). The overexpression of cardiac SERCA (103, 104) in mouse was found to be associated with an increased maximum velocity of Ca^{2+}-uptake (V_{max}) as well as increased rate of contraction (+dP/dt) and rate of relaxation (-dP/dt). These results demonstrate that the enhancement in SERCA pump level can increase the cardiac contractile function by increasing the SR Ca^{2+} transport activity (103).

Phospholamban (PLB) is a phosphoprotein (25 kD) which modulates the function of SERCA (105). There is only one kind of PLB present in cardiac muscle and slow-twitch skeletal muscle (67). In its unphosphorylated form, PLB binds to SERCA and prevents the transport of Ca^{2+} into the SR lumen. Phosphorylation of PLB by cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) blocks the interaction between PLB and SERCA and thus increases the rate of Ca^{2+}-uptake into the SR. Calmodulin, another Ca^{2+}-binding protein, is involved in the phosphorylation of PLB and plays a role in Ca^{2+}-transport (1, 75, 106). Calsequestrin (CQS) is a Ca^{2+}-binding protein in SR with a molecular weight of 53 kD (1). It is confined to regions in the SR that store and release Ca^{2+} and is closely associated with the t-tubules and the non-junctional SR (91). In the cardiac SR, there is another Ca^{2+}-binding protein, calreticulin (67), whose function still remains to be defined clearly.

5. SR Alterations and Changes in Gene Expression in CHF

The intracellular Ca^{2+}-transients and cardiac contractions are markedly prolonged in the failing human heart (107, 108). Ca^{2+} homeostasis was disturbed in failing human ventricular myocytes and this was reflected by smaller and more slowly decaying

Ca^{2+}-transients. Ca^{2+}-release from the SR was decreased while Ca^{2+}-influx via the reverse-mode of the Na^{+}/Ca^{2+} exchanger was increased (109). It was identified that both the density and kinetics of L-type Ca^{2+} current were not significantly different in the post-MI remodeled rat myocytes compared with control (110). On the basis of several such studies, it was concluded that SR changes occur in human heart with CHF (111) (Table 1).

The $[Ca^{2+}]_i$ was reduced due to a decrease in Ca^{2+}-release from the SR in the infarcted rat heart (112, 113). By using skinned fibers, it was shown that the amount of Ca^{2+} released from SR was decreased upon stimulation with caffeine in CHF following MI in rats. Total RYR density (B_{max}) was reduced after 8 weeks of MI, while the receptor affinity (K_d) did not change. In addition, it was found that the decreased Ca^{2+}-release in the failing heart was due to the down-regulation of the SR Ca^{2+}-release channel (36, 43). In contrast, it has been reported that the density of RYR in cardiomyocytes from the MI rat heart is increased or unchanged (12). Nonetheless, it was evident that Ca^{2+} sequestration by SERCA was impaired in the failing human myocardium (33, 75) as well as in the MI rats (112-114). It has been identified that the SERCA protein concentration was decreased and the ratio of SERCA to PLB or CQS was reduced in the human failing heart (115); however, some investigators have reported that the SERCA concentration did not change in the failing heart (116). A depression in the SERCA activity in CHF can result in the inability of myocardium to relax fully. For example, the protein level and the activity of SERCA begin to decrease in rats 4 weeks after MI and by 8 weeks, these dropped to about 79% and 35% of control values, respectively (29). On the other hand, in the heterozygous mice with a null

Table 1. Alterations in the cardiac SR Ca^{2+}-regulation protein contents in patients with heart failure

First Author & References	Tissue Source	RYR	SERCA2a	PLB	CQS
Movsesian (147)	CHF heart before transplantation	ND	—	—	—
Schwinger (116)	CHF heart before transplantation	ND	—	—	—
Hasenfuss (74, 148)	CHF heart of dilated and ischemic cardiomyopathy	ND	↓	↓	—
Linck (149)	CHF heart before transplantation	ND	↓	↓	ND
Meyer (115)	CHF heart of dilated cardiomyopathy	—	↓	—	—

ND - not detected; — : no change; ↓ - decrease; RYR - ryanodine receptors; SERCA2a - sarcoplasmic reticular Ca^{2+}-pump; PLB - phospholamban; CQS - calsequestrin

mutation in SERCA2, both SERCA2a protein level and the rate of SR Ca^{2+}-uptake were reduced by 35% on the appearance of CHF (97). In the cardiomyocytes isolated from the non-infarcted region of the rat LV 6 weeks after MI, the peak L-type Ca^{2+} current was not significantly changed but peak Ca^{2+}-transients recorded by Fura-2 were decreased by 19% (41). It was also found that when SERCA in the infarcted heart was inhibited by thapsigargin (1μM), no further change of relaxation time was evident. These observations indicated that the function of SERCA2a was greatly down-regulated in CHF (41).

The depressed SERCA activity in SR membranes from the viable LV 16 weeks after MI was seen at different Ca^{2+}, K^+ and ATP concentrations; however, no alterations in the affinities of the enzyme for Ca^{2+} and ATP were observed. In addition, the SR phosphorylation (^{32}P incorporation) was reduced in the presence of Ca^{2+}-calmodulin or cAMP-dependent protein kinase (PKA) (40). Other investigators showed a decrease in Ca^{2+}-pump ATPase activity in rats after MI (117). These results reveal that the function of SERCA2a is decreased in rats after MI and this may cause alterations of myocyte contraction in the failing hearts due to MI. On the other hand, despite a comparable degree of cellular hypertrophy and more severe hemodynamic decompensation, the expression of SERCA and PLB were not changed in MI-induced heart failure (12) or in some clinical studies (116). CQS, unlike PLB, was unchanged during the development of CHF following MI (Tables 1 and 2).

There is an immediate induction of the fetal/embryonic transcriptional gene program in the heart following MI in rats which may precede myocyte hypertrophy and persist longer

Table 2. Alterations in cardiac SR Ca^{2+}-regulation protein contents in animal models of heart failure

First Author & References	Tissue Source	RYR	SERCA2a	PLB	CQS
Hisamatsu (62)	Rats after 12 weeks of aortocaval shunt	↓	↓	ND	ND
Yue (12)	Isolated myocytes of rats 6 weeks after MI	ND	—	—	ND
Qi (60)	8 weeks after abdominal aortic coarctation	ND	—	ND	ND
	16 weeks after abdominal aortic coarctation	ND	↓	ND	ND
Kiss (53)	Guinea pig after 8 weeks of aorta banding	ND	↓	↓	ND
Liu (150)	8 weeks after abdominal aorta banding in rats	ND	↓	ND	ND
O'Rourke (65)	Rapid pacing dogs	ND	↓	↓	ND
Periasamy (97)	Heterozygous mice with null mutation SERCA	ND	↓	ND	ND
Takeishi (140)	Aortic-banded guinea pigs	ND	↓	↓	ND

All abbreviations are same as in Table 1.

than in the pressure-overload hearts (12). Abnormalities in SR Ca^{2+} transport in CHF have been suggested to be due to changes in gene expression for the SERCA protein in different models of heart failure (1). The SERCA2 gene is expressed in high amounts in the heart and encodes the mRNA for two different isoforms (99). The cardiac SERCA2 mRNA level drops significantly by 20% and 35% at 4 weeks and 16 weeks after MI in rats, respectively. The alterations in mRNA and SERCA protein expression were comparable while the SERCA activity was greatly reduced (Table 3). These results demonstrate that changes in SERCA may be due to alterations at both the gene transcriptional level and the post-transcriptional level (mRNA stability) (29). On the other hand, when mRNA level of the cardiac SERCA was reduced by 45% in heterozygous mice with a null mutation of SERCA, the protein level and Ca^{2+}-uptake velocity were reduced markedly (97).

6. Presence of RAS in the Cardiovascular System

RAS is one of the major systems regulating cardiovascular function and includes different components. Angiotensinogen, a 452 amino acid globular glycoprotein of 55-56 kD, is the precursor of Ang II. It is mainly localized in the pericentral zone of the liver lobules. The plasma is the major reservoir of angiotensinogen and thus it is the major determinant of RAS activity (118). Renin, a glycoproteolytic single-chain aspartyl protease of 37-40 kD, is highly specific for its substrate, angiotensinogen, and is generated in the juxtaglomerular cells of the afferent arterioles of the kidney. The renin mRNA is found in almost all organs of the body, but its level in the heart is rather low. Renin cleaves a

Table 3. Alterations in cardiac mRNA levels for SR Ca^{2+}-regulation proteins in congestive heart failure

First Author & References	Tissue Source	RYR	SERCA2a	PLB	CQS
Mercadier (151)	CHF heart before cardiac transplantation	ND	↓	ND	ND
Linck (149)	CHF heart before transplantation	ND	—	—	ND
Schwinger (116)	CHF heart before transplantation	ND	↓	—	ND
Arai (154)	CHF before cardiac transplantation	↓	↓	↓	—
Feldman (152)	Endomyocardial biopsies of human CHF heart	ND	ND	↓	ND
Feldman (153)	Failing LV of rats 20 weeks after aortic banding	ND	↓	—	ND
Yue (12)	Isolated myocytes of rats 6 weeks after MI	ND	—	—	ND
Iijima (117)	LV of rats 4 weeks after MI	—	↓	—	—
Qi (60)	8 weeks after abdominal aortic coarctation	ND	↓	ND	ND
	16 weeks after abdominal aortic coarctation	ND	↓↓	ND	ND
Periasamy (97)	Heterozygous mice with null mutation SERCA	ND	↓	ND	ND
Liu (150)	8 weeks after abodminal aorta banding in rats	ND	↓	ND	ND

All abbreviations are same as in Table 1.

leucine-valine bond in the N-terminal region of angiotensinogen for the generation of angiotensin I (Ang I) (118, 119). Ang I is composed of 10 amino acids and does not have any significant effect of its own; however it is converted by angiotensin converting enzyme (ACE) to Ang II. ACE, a dipeptidyl carboxypeptidase, is a member of the family of zinc metallopeptidases, derived from the lung and other organs in the body. ACE is predominately attached to endothelial cells (2) and in addition to converting Ang I to Ang II, it inactivates a well known vasodilator, bradykinin. Ang II plays a major role and shows almost all the effects of RAS when it combines with its receptors. An alternative pathway for Ang II production in the human heart is via chymase (a chymotrypsin-like proteinase), which is not affected by ACE inhibition (2). Ang II receptor heterogeneity has been defined primarily by the use of non-peptides to show that there are two distinct types of ACE receptors. Ang II type 1 (AT_1) receptor is composed of 359 amino acids with AT_{1A} and AT_{1B} subtypes (120). It mediates most of the physiologic and pathophysiologic effects of angiotensin II and involves the translocation of protein kinase C (PKC) from the cytosol to the membranes and is blocked by the specific AT_1 receptor antagonist, losartan (2, 121). The Ang II type 2 (AT_2) is present in the heart during fetal development and is blocked by PD123177. Although in adult ventricular myocytes, AT_1 receptor subtype plays a major role (2), it was found that inhibition of AT_2 receptors by PD123319 could amplify the immediate LV growth response to Ang II with enhanced PKC translocation to membranes and reduced LV cGMP content (122).

Ang II receptors are widely distributed throughout the heart and each receptor subtype

accounts for about 50% of the specific binding by losartan or PD123177. The density of Ang II receptors is high in the atrioventricular node (123). In the human atria, the ratio of AT_2:AT_1 receptors is about 2:1, but in rats, the ratio is about 69%:31%. However, most of the Ang II effects are mediated by the AT_1 receptor only (121). The stimulation of AT_1 receptors activates phospholipase C (PLC) to produce phosphatidylinositol 4,5-bisphosphate, then forming diacylglycerol (DAG) and IP_3 which induces SR Ca^{2+}-release and increases contractility (72, 124). On the other hand, DAG activates PKC and thus stimulates cardiac growth (125). Ang II was observed to increase the $[Ca^{2+}]_i$ in rat cardiomyocytes as measured by Fura-2/AM fluorescence spectroscopy; this increase is dependent on the concentration of Ang II (0.01 to 10 μM) (124). In cardiac fibroblasts, Ang II decreased the activity of metalloproteinase I, which is the key enzyme for interstitial collagen degradation (11). The existence of tissue RAS in the heart was identified by showing the presence of mRNA for angiotensinogen, ACE and prorenin in cardiomyocytes (2). It should be mentioned that Ang II has multiple effects on the heart and periphery, acting directly or indirectly on myocytes for the regulation of growth, vascular resistance and contractility. The effects of Ang II are mediated via its receptors, which are located on ventricular and smooth muscle cells and are linked to guanine nucleotide-binding proteins (G-proteins) that control the generation of various downstream second messenger pathways (2). Mechanical stretch has been shown to initiate the release of Ang II which acts as an initial mediator of the hypertrophic response in cardiac myocytes (126).

7. Role of RAS in MI and CHF

A reduction of renal blood flow is detected by sensory receptors in the renal arterioles and this initiates the release of renin from the kidney during the development of CHF. The formation of Ang II attenuates the baroreceptor sensitivity and in fact patients with CHF exhibit an excessive activation of the RAS at rest or during exercise (14). In the paced rabbit, the elevated levels of plasma Ang II and decreased formation of nitric oxide were shown to be necessary for sustained increases in the sympathetic nerve activity (127). In the rat model of MI, the angiotensinogen mRNA levels were significantly elevated in the non-infarcted portion of the LV at 5 days after infarction, and showed a significant correlation with infarct size and increased LVEDP. However, such alterations disappeared at 25 days after MI (128). In this experimental model of MI, an increased expression of c-myc and c-jun was demonstrated in the viable myocytes of RV and LV 2 to 3 days after MI while the myocyte volume increased (2). On the other hand, the Ang II receptors, mainly located on the non-myocytes and fibroblasts (121), were found elevated in rats after MI; the AT_{1A} mRNA level was increased while the AT_{1B} mRNA did not change (117). The cardiac ACE activity was increased markedly in the infarcted area and moderately in the viable hypertrophied myocardium without any alteration of the affinity to the inhibitors (129). The up-regulation of ACE mRNA has been shown in experimentally induced pressure overload and post-infarction heart failure (2). In the CHF rats after MI, the ventricular myocytes were found to possess the AT_1 receptor subtype exclusively whereas the Ang II-stimulated phosphoinositol turnover was enhanced by 3.7-fold and 2.5-fold in the LV and RV

myocytes, respectively (130). It should be pointed out that RAS is involved in pathologic myocardial fibrosis because Ang II was reported to increase the collagen synthesis and inhibit the collagenase activity in cultured adult cardiac fibroblasts (2). From this evidence, pharmacological interference suggests that the RAS may be considered to be of potential physiological and clinical importance for remodeling of the infarcted LV. In fact, inhibition of the RAS has become the therapeutic strategy used in clinical practice. The inhibition of the RAS can be achieved either by reduction of Ang II by using ACEI or by blockade of the Ang II receptors (131).

8. Modification of CHF by Angiotensin Converting Enzyme Inhibitor (ACEI)

The therapy of CHF with ACEI can be instituted at early stages of CHF (132) but these agents have also been shown to exert a marked beneficial effect even when therapy was initiated at late stages of CHF (133). However, the mechanisms of action, time frame for the beneficial effects and duration of therapy with ACEI are still unknown (6). It is pointed out that ACEIs are considered to act via a decrease in the production of Ang II which in turn results in an improvement of cardiac function and clinical outcome (134). In CHF after MI, ACEIs have been demonstrated to improve heart function and increase survival rate in rats and in humans; the clinical and hemodynamic benefits of ACEIs in CHF in terms of improving exercise tolerance have now been well accepted (135, 136). Clinical trials of ACEI, such as enalapril (ENP), show reductions in morbidity and mortality of patients with CHF or after MI (Table 4) (136, 137). In infarcted rats, ACEI reduced ventricular

Table 4. Major clinical trials of enalapril for the treatment of congestive heart failure

Clinical Trial & References	Patients	Time	Dose	Effects
CONSENSUS I (155)	CHF not responsive to conventional therapies	–	–	reduced mortality in the elderly CHF population
CONSENSUS-II (156)	6, 090 post-MI	24 hr-180 days	–	no improvement of survival in first 180 days after MI
VheFT II (157)	mild to moderate CHF	2 years	20 mg/day	improved survival; no great improvement of EF
SOLVD (158, 159)	asymptomatic and mild CHF	–	10 mg b.i.d.	improved clinical symptoms
SOLVD (158)	–	–	11.2 mg/day	reduced mortality and recurrence of MI

CHF: congestive heart failure; CONSENSUS: Cooperative New Scandinavian Enalapril Survival Study; EF: ejection fraction; MI, myocardial infarction; SOLVD: Studies of Left Ventricular Dysfunction; VHeFTII: Veteran's Administration Cooperative Vasodilator Heart Failure Trial II.

volume and produced more favorable ventricular performance. ACEIs not only reduced the LV filling pressure and ventricular distension but also reduced the dilatation of LV (Table 5) without reducing the infarct size (43). Irrespective of early versus delayed ACE inhibition in rats after MI, LVSP, LVEDP and central venous pressures were reduced; LV weight, LV cavity size and LV collagen density were also decreased (133). In clinical studies, long-term therapy with ACEIs can favorably alter the loading conditions on the LV and reduce the progressive ventricular enlargement; this attenuation of the ventricular enlargement is associated with a prolongation in survival (2, 5, 6, 15, 17, 26, 136).

ACEIs are considered to affect heart failure by a combination of neurohormonal, hemodynamic, left ventricular structural remodeling and other effects (136). First, ACEIs directly decrease the circulating and tissue effects of Ang II by reducing its production. ACEIs may suppress the sympathetic-mediated ventricular dilation caused by increased cardiac workload or direct trophic effects on the myocardium (2, 6, 136). These drugs may also diminish the level of plasma aldosterone and prevent secondary sodium and volume retention (2, 6, 136). The circulatory catecholamines are reduced, while the degradation of bradykinin is diminished by ACEIs (2, 136). However, ACEIs do not completely inhibit the production of Ang II which can also be produced through non-ACE pathway (125). Secondly, these drugs can induce veno- and arteriolar dilation resulting in improved stroke volume and reduced atrial and ventricular diastolic volume without chronotropic stimulation (2, 6, 136). In patients treated with captopril, ischemia-related events were reduced during the first 3 to 12 months after MI but there was rebound phenomenon after the withdrawal of

Table 5. Major animal experiments for ENP in the treatment of MI and/or CHF

First Author & Reference	Model	Duration	Dose	Effects
Sanbe (139)	MI rat	2^{nd} - 12^{th} weeks	10 mg/kg/day	MAP ↓, LVEDP ↓, CO ↑, metabolism ↑
Sanbe (28)	MI rat	2^{nd} - 12^{th} weeks	10 mg/kg/day	CO ↑ β_1-adrenergic receptor density ↑
Schieffer (160)	MI rat	2^{nd} - 7^{th} weeks	0.5 mg/kg/day	coronary vascular resistance ↓ interstitial fibrosis ↓

↑ - increased; ↓ - decreased; MI - myocardial infarction; CHF - congestive heart failure; MAP - mean arterial pressure; LVEDP - left ventricular end diastolic pressure; CO - cardiac output

ACEI treatment. Such benefits are sustained with continued therapy in CHF; however, withdrawal can result in exacerbation of disease (138). Thirdly, ACEIs can decrease the transmural wall stress, compensatory dilatation and compensatory increase of end-diastolic and end-systolic volume. In addition, ACEIs directly or indirectly alter remodeling process of the LV and improve the coronary flow distribution in both surface and transmural areas (2, 6, 136). On a long-term basis (6 to 8 weeks), treatment with ACEI also reduces the abnormal accumulation of myocardial collagen in MI rats (2).

Several studies have been carried out for ACEI therapeutic effects (Table 5). Long-term therapy with ENP in MI rats was observed to decrease MAP and LVEDP. ENP also prevents the reduction in cardiac output (CO) and stroke volume indices in addition to reducing the mitochondrial oxygen consumption by the viable LV and RV. The underlying mechanism of therapy is attributable to recovery or preservation of mitochondrial function, reduction in preload and attenuation of the decrease in β_1-adrenergic receptor density (139). In the pressure-overload rat model of aortic-banding, ACEI therapy with ramipril improved LV function and prevented the down-regulation of Ca^{2+}-cycling protein expression while the reduced PKC levels were also attenuated (140). It was found that the depression in aortic flow, CO and RYR density in SR (43) was attenuated after treatment of infarcted rats with trandolapril. The major side effects of ACEIs are cough, renal dysfunction and first dose hypotension, which are due to ACEI-induced bradykinin formation but there are still some patients having poor LV function despite ACEI treatment (134).

9. Modification of CHF by Ang II Receptor Antagonist

An orally active antagonist of angiotensin receptors, losartan (LOS), was first introduced into clinical practice at the beginning of this decade. LOS is a non-peptide AT_1 subtype Ang II receptor antagonist without any agonist actions (20, 121). Ang II receptor antagonists can directly block the action of Ang II by suppressing the receptors. LOS has not only been utilized as a tool to investigate the role of Ang II in CHF but also has been introduced as a new CHF therapy. The blockade of RAS by LOS was more specific than that by ACEIs, and was reported to show different effects (141). LOS is devoid of the side effects caused by ACEI, especially due to an increase in the level of bradykinin (125). LOS alone improves the symptoms in CHF after MI or other diseases (142). A comparison of the efficacy, tolerability and effect on mortality of long-term Ang II receptor blockade with LOS is given in Table 6 (143). Furthermore, LOS was reported to enhance the exercise capacity and alleviate the clinical symptoms (144) in CHF patients who were severely symptomatic even during treatment with optimal doses (maximally recommended or tolerated) of ACEIs. In normal rat myocytes, LOS was shown to block the increase of $[Ca^{2+}]_i$ and cell beating stimulated by Ang II (124). It was demonstrated that either LOS alone or combined with ENP could limit cardiac hypertrophy in MI rats; however, LOS did not have marked effects on non-myocyte cellular proliferation (145). Furthermore, there is some controversial data that LOS (10 mg/kg/day) did not exert marked beneficial effects on cardiac function in MI rats (131) and did not improve the cardiac output in pacing dogs (146). Nonetheless, there is an ample body of evidence to show the beneficial effects of LOS and other AT_1 receptor antagonists in different types of heart failure.

Table 6. Major animal experiments for LOS treatment of MI and/or CHF

First Author & Reference	Animal Model	Method	Dose	Effects
Regitz-Zagrosek (142)	human CHF	oral	5-150 mg once	MAP ↓, PCWP ↓, cardiac index ↑
	human CHF	oral	5-15 mg/day for 12 weeks	MAP ↓, PCWP ↓
Raya (161)	MI rats	oral	40 mg/kg/day	MAP –, LVEDP ↓
Smits (141)	MI rats	oral	15 mg/kg/day	CO – left ventricular hypertrophy ↓
Murakami (146)	pacing dog	venous	1.1 mol/kg	No effects on cardiac output
Liu (150)	pressure overload rat	oral	20 mg/kg/day for 8 weeks	SERCA2 ⇑
Schieffer (160)	MI rat	2^{nd} - 7^{th} weeks	3 mg/kg/day	coronary vascular resistance ↓ interstitial fibrosis ↓

⇑ - partial increase compared to decrease in the sham operated animal; PCWP: pulmonary capillary wedge pressure. All other abbreviations are the same as in Table 5.

II. STATEMENT OF THE PROBLEM AND HYPOTHESIS TO BE TESTED

MI is known to result in cardiac remodeling and subsequent CHF but the exact mechanisms of these pathophysiological alterations are still not fully understood. Ca^{2+}-handling abnormalities in cardiomyocytes lead to cardiac dysfunction in CHF. Defects in L-type Ca^{2+}-channel proteins or activity during CHF are not firmly established (162). However, the Ca^{2+}-regulating function of the SR, which is central for E-C coupling is depressed in CHF after MI. It is thus reasonable to assume that the SR Ca^{2+}-regulating proteins may change in density and/or activity. The work from several laboratories has indicated that defects in the SR membrane from MI are due to changes in the expression of genes specific for the SR Ca^{2+}-regulating proteins. Both the mRNA level and protein content of RYR, SERCA2 and PLB in the MI group are decreased compared with sham group, while CQS remained unchanged.

In addition, it is well accepted that RAS plays an important role in LV remodeling and CHF after MI. RAS affects not only the structural remodeling of LV, but also the myocyte function in MI; however, the mechanism of RAS function in CHF is still poorly understood. In clinical studies and animal experiments, blockade of RAS has become the major therapeutic method in MI-induced CHF, but the subcellular and molecular mechanisms of RAS blockade are not clear. The beneficial actions of RAS blockade on cardiac function in CHF may be due to its ability to prevent membrane remodeling in the failing heart. Accordingly, it is hypothesized that the blockade of RAS may attenuate the defects in SR

Ca^{2+}-regulating proteins in CHF induced by MI.

It has already been shown that ENP and LOS have comparable efficacy and tolerability in patients with moderate and severe CHF (143) and reduce cardiac hypertrophy in MI rats (160). In order to investigate the therapeutic mechanism of RAS blockade, changes in the steady state mRNA levels and protein contents of RYR, SERCA, PLB and CQS were evaluated upon treatment of the MI animals with an ACEI (ENP) or AT_1 receptor antagonist (LOS). Therefore, two series of studies were designed to test the effects of RAS blockade on SR Ca^{2+}-regulating proteins in MI-induced CHF in rat. In the first series of experiments, ENP was utilized, whereas LOS was used in the second series of experiments. This investigation is expected to provide comprehensive information regarding the pathogenesis of SR dysfunction and pathophysiology of CHF after MI in the absence and presence of RAS blockade.

III. METHODS

1. Experimental Model

MI was produced in male Sprague-Dawley (SD) rats (body weight 175-200 g) by surgical occlusion of the left coronary artery as described previously (23-25, 40). After rats were anesthetized with isofluorane, different leads of the electrocardiogram (EKG), I, II, III, aVR, aVL and aVF were recorded. The skin of the animal was incised along the left sternal border and the heart was exposed by a left thoracotomy performed by cutting the third and fourth ribs. The pericardial sac was perforated and the heart was extruded via the intercostal space, the left coronary artery was ligated 2-3 mm from the origin with a suture (6-0 silk), the heart was repositioned in the chest and the air in the thoracic cavity was removed by using a syringe after closing the chest with a purse-string suture. During the whole period of operation, ventilation of the lungs was maintained by positive-pressure inhalation of 95% O_2 and 5% CO_2 mixed with isofluorane. Sham-operated animals were treated similarly except that the coronary suture was not tied. The mortality of all animals operated upon in this fashion was about 30% within 48 hours. All experimental protocols of animal studies were approved by the Animal Care Committee of the University of Manitoba, following guidelines established by the Medical Research Council of Canada.

2. Protocol for Drug Therapy

All rats received standard care, kept at 12 hr day/night cycle and were fed rat chow and water *ad libitum*. There are two types of experiments with 4 groups in each series. In

the first series of experiments, there are sham, sham treated with ENP, MI, and MI treated with ENP groups of animals. The second series of experiments were similar to the first except that LOS was utilized instead of ENP. Twenty-one days after the operation, EKGs was recorded from all the rats. Then ENP (10 mg/kg/day for 4 weeks) or LOS (20 mg/kg/day for 4 weeks) was given orally by a gastric tube. Therapy was started at 3 weeks after surgery based on the previous study suggesting that the myocardial infarct was completely healed 3 weeks after occlusion of the coronary artery in rats. The EKG recording, hemodynamic assessment and biochemical studies in sham-control and infarcted animals with or without drug treatments were carried out at 7 weeks after the surgery. ENP and LOS were supplied by MERCK Research Laboratories, Rahway, NJ, USA.

3. Hemodynamic Measurements

The animals were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). After exposing the right carotid artery, a cannula with a microtip pressure transducer (model SPR-249, Millar Instruments, Houston, TX) was introduced into the artery through a proximal arteriotomy. The MAP was measured at this point and the catheter was advanced to enter the left ventricle. The catheter was fixed with a silk ligature around the artery and hemodynamic recordings were taken via software (AcqKnowledge for Windows 3.0, Harvard Apparatus, Montreal, Canada). The LVSP, LVEDP, heart rate, rate of pressure development (+dP/dt) and rate of pressure decay (-dP/dt) were measured in these anesthetized animals. After the hemodynamic measurements, the

hearts were removed and the right and left ventricles as well as the scar tissue were dissected and weighed. In addition, the lungs and livers from all the animals were removed and weighed immediately. Upon drying the lungs and livers in the oven, these were weighed again. The experimental rats with large infarct scar (30-45% area) of the left ventricle were employed in the study. 10 to 12% of the infarcted animals with or without drug treatments showed infarct size < 25% of the left ventricle; these animals were not included in this study.

4. EKG Measurement and Analysis

The EKG recordings of six groups of rats were performed in stablilized animals under isoflurane anesthesia with EC-60 Cardiac and Respiratory monitor (Silogic International Limited, Surrery, U.K.). Six-lead electrocardiograms (EKG: limb leads I, II, III, aVR, aVL and aVF) were recorded simultaneously at four time points: before opening of the chest, after the closure of chest, just before starting the therapy (3 weeks after surgery) and 7 weeks after the surgery. According to the modified method of QRS scoring system in humans (9, 163), only the amplitude of Q wave in lead I, -aVR and aVL were measured in mV to estimate the gross size of MI in the rats.

5. RNA Extraction

Total RNA was extracted from the viable cardiac tissues by the procedure of Chomczynski and Sacchi as described previously (164). Briefly, cardiac tissue was rapidly excised, the atria were removed, and the left ventricular viable tissue, including the septum,

was washed twice with a solution containing 10 mM 3-[N-morpholino]propanesulfonic acid (MOPS) and 10 mM sodium ethylenediaminetetraacetate (EDTA). Tissue samples were then quickly frozen and stored in liquid nitrogen (-80°C). The frozen ventricular tissue was ground with mortar and pestle in liquid nitrogen and the powdered samples were suspended in 4 ml Solution D [4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0) 0.5% N-lauroylsarcosine, 0.1M 2-mercaptoethanol]. Then the tissue homogenates were treated with 0.1 volumes of 2M sodium acetate (pH 4.0), equal volumes of water-saturated phenol (pH 7.4), and 0.2 volumes of chloroform-isoamyl alcohol mixture (49:1) and mixed by inversion. After the mixture was cooled on ice for an additional 15 min, samples were centrifuged at 6,000 × g for 20 min at 4°C. The RNA-containing aqueous phase was transferred to a fresh tube, mixed with an equal volume of isopropanol and placed at -20°C for 60 min. RNA was sedimented at 10,000 × g for 20 min and resuspended in solution D, and then again precipitated with an equal volume of cold isopropanol and placed at -20°C for 30 min. Afterwards, the ventricular RNA pellets were washed twice with 75% ethanol and sedimented; the ethanol solution was decanted and finally vacuum dried. RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water and the concentration of nucleic acid was calculated from the absorbance at 260 mm prior to size fractionation.

6. Northern Blot Analysis

Steady-state levels of mRNA were determined by Northern hybridization analysis. Twenty μg of total RNA was denatured in 50% formamide, 7% formaldehyde, 20 mM

MOPS (pH 7.4), 2mM EDTA (pH 8.0), 0.1% SDS and electrophoresed in a 1% agarose/formaldehyde gel to size fractionate the mRNA transcripts. The fractionated RNA was transferred onto a 0.45 μm positively charge-modified nylon filter (NYTRAN Maximum Strength Plus, Schleicher and Schuell, Keene, NH, USA) by capillary action. The filter was removed after 24 hr and RNA was covalently crosslinked by UV radiation (UV Stratalinker 2400, Stratagene). Blots were prehybridized in a mixture of 50% formamide, 10% Denhardt's solution, 1% SDS, 0.2 mg/ml denatured salmon sperm DNA, 10 mM EDTA (pH 8.0), 25% " 4× RNA" solution [3 M NaCl, 0.6 M Tris-HCl (pH 7.5), 0.18 M NaH_2PO_4, 0.24 M Na_2PO_4, 0.1 M $Na_4P_2O_7$] at 42°C for 6-16 hr. Membranes were hybridized for 6 to 16 hr at 42°C in the presence of ^{32}P labeled specific probes with a specific activity > 10^9 cpm per μg DNA. The following cDNA and oligonucleotide probes were utilized: a) RYR: a 2.2 kb cDNA fragment of the rabbit cardiac RYR (courtesy of Dr. D.H. MacLennan, University of Toronto, Toronto, Canada); b) SERCA2: a 0.762 kb cDNA fragment of the rabbit cardiac Ca^{2+}-ATPase (courtesy of Dr. A.K. Grover, McMaster University, Hamilton, ON, Canada); c) PLB: a 0.153 kb cDNA fragment of the rabbit cardiac PLB (courtesy of Dr. D.H. MacLennan, University of Toronto, Toronto, Canada); d) CQS: a 2.5 kb cDNA fragment of the rabbit cardiac CQS (courtesy of Dr. A. Zilberman, University of Cincinnati, Cincinnati, OH, USA); e) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): a 1.2 kb cDNA fragment of the human GAPDH (American Type Culture Collection, Rockville, MD, USA) and f) 18S: a 24 base oligonucleotide probe of rat 18S ribosomal RNA. The filter was then washed in 1 × SSC /1% SDS inside an INNOVA 4000 incubator (New Brunswick Scientific,

Canada) oscillating at a rate of 64 rotations per min. After washing, the membrane was exposed to X-ray film (Kodak X-OMAT) with two intensifying screens at -70°C. The mRNA bands after autoradiography were quantitated by scanning densitiometry with the use of GS-670 (Bio-RAD Company, Mississauga, ON, Canada). All mRNA signals were normalized to the respective GAPDH signal.

7. Isolation of SR Membrane

SR membrane fraction was isolated according the method in our laboratory (49). In short, viable left ventricular tissue was homogenized in a polytron (Kinematica, Switzerland) in a medium containing 10 mM $NaHCO_3$, 5 mM NaN_3, and 15 mM Tris-HCl (pH 6.8) at the speed of 12,000 rpm for 45 sec. The homogenate was centrifuged at 10,000 g for 20 min. The supernatant was centrifuged at 40,000 g for 30 min. The pellet was suspended in 0.6M KCl and 20mM Tris-HCl (pH 6.8) to solubilize the contractile proteins and again centrifuged at 40,000 g for 45 min. The final pellet was washed and suspended in 0.25 M sucrose and 10 mM histidine and stored at -70°C. The protein concentration was evaluated by using the Lowry's method.

8. Western Blot Analysis

The relative contents of SR RYR, SERCA2, PLB and CQS proteins were determined by running 5-12% mini gel with a 4% stacking gel of sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. The SR vesicles (1 mg/ml) were

added to the SDS-PAGE loading buffer (0.25 M Tris-HCl (pH 6.8), 8% (w/v) SDS, 45% glycerol, 20% β-mercaptoethanol and 0.006% bromophenol blue) in a ratio of 3:1. The sample loads for each group were of the same volume (10 µl in each well, 20 µl in RYR experiment). The SDS-PAGE was carried out at 200 voltage for 45-60 min. The separated proteins were then electroblotted to immobilon-P transfer membrane (Millipore Corporation, Bedford, MA, USA) in transfer buffer containing 25 mM Tris-HCl, 120 mM glycine and 20% methanol (v/v) at 0.5 mA. The transferred membranes were shaken for 2 hr in blocking buffer containing TBS (10 mM Tris, 150 mM NaCl) and 5% fat-free powdered milk. The membranes were incubated for 1 hr at room temperature with either a monoclonal mouse anti-SR RYR antibody (1:1,000 Research Diagnostics Inc., NJ, USA), SERCA antibody (1:2,000 Affinity Bioreagents Inc., Golden, CO, USA), mouse anti-SR PLB antibody (1:5,000 Upstate Biotechnology, Lake Placid, NY, USA) or anti-SR CQS antibody (1:3,000 Upstate Biotechnology, Lake Placid, NY, USA). The membranes were subsequently incubated for 40 min with secondary antibody (IgG antibody 1:3,000, Amersham Corporation, Arlington Heights, IL, USA). Finally, the membranes were incubated with strepdavidin conjugated horseradish peroxidase (1:5,000, Amersham Corporation, Arlington Heights, IL, USA) for 30 min at room temperature. The blots were rinsed in the TBST (TBS and 0.2% Tween 20) buffer 3 times (10 min each time) between each of the preceding steps. For chemiluminescent detection, the membrane sheets were dipped into the luminal substrate solution (Amershan Corporation, Arlington Heights, IL, USA) and the chemilumigrams were developed on ECL-Hyperfilm (Amersham Corporation) to visualize SR RYR, SERCA2, PLB

and CQS bands. The bands were analyzed by the model GS-670 Imaging Densitometer (Bio-Rad Company, Mississauga, ON, Canada) with the Image Analysis Software Version 1.0 and were expressed in relation to control values.

9. Statistical Analysis

All values are expressed as mean ± S.E. The difference among control and experimental groups were calculated using the analysis of variance followed by Scheffe's test. Significant differences among groups were defined by a probability of $P< 0.05$.

IV. RESULTS

1. Effects of ENP on LV and SR After MI

a. General characteristics

General characteristics of the coronary artery ligated and sham-operated rats are shown in Table 7. ENP (10 mg/kg/day) was given at 21 days after the operation. Animals were divided into four groups: sham, sham with ENP treatment (sham + ENP), left coronary artery ligated (MI) and coronary ligated plus ENP treatment (MI + ENP). There was no significant change of body weight and liver wet/dry weight ratio between each group at different time points: pre-operation, 3 weeks or 7 weeks after MI. However, the heart weight (including LV, RV and scar), heart/body weight ratio and the lung wet/dry weight ratio were significantly increased in the MI animals. These changes in the MI animals were partially prevented upon treatment with ENP. The scar weight in the MI group was approximately 20% of the left ventricle and there was no scar in the left ventricle of sham operated rats. No difference of scar weight was found between the MI animals with and without ENP treatment.

b. Hemodynamic study of LV function and EKG measurements

Hemodynamic assessment showed a significant decrease in +dP/dt and -dP/dt, and an increase in LVEDP of the MI rats 7 weeks after the operation; no difference in heart rate, LVSP or MAP between each group was observed (Table 8). Treatment of MI animals with ENP partially normalized the hemodynamic alterations.

Table 7. General characteristics of myocardial infarcted rats with or without enalapril, losartan treatment for 4 weeks starting at 3 weeks after coronary occlusion.

	Sham	Sham + ENP	Sham + LOS	MI	MI + ENP	MI + LOS
BW_0	265 ± 14	256 ± 7	264 ± 14	262 ± 18	266 ± 16	249 ± 10
BW_3	404 ±21	390 ± 8	389 ± 17	390 ± 16	412 ± 18	398 ± 15
BW_7	495 ± 9	479 ± 9	491 ± 20	495 ± 21	500 ± 25	489 ± 24
Heart wt (mg)	1158 ± 45	1183 ± 43	1091 ± 32	1537 ± 30*	1314 ± 17*#	1333 ± 27*#
LV scar wt (mg)	ND	ND	ND	221 ± 24	219 ± 23	235 ± 15
Heart wt/BW ratio (mg/g)	2.33 ± 0.12	2.47 ± 0.08	2.22 ± 0.1	3.10 ± 0.05*	2.63 ± 0.06*#	2.72 ± 0.08*#
Lung wet/dry wt ratio	4.28 ± 0.1	4.71 ± 0.22	4.37 ± 0.16	5.39 ± 0.2*	4.87 ± 0.29#	4.92 ± 0.31#
Liver wet/dry wt ratio	3.14 ± 0.04	3.28 ± 0.06	3.29 ± 0.11	3.28 ± 0.07	3.44 ± 0.03	3.34 ± 0.08

Values are mean ± S.E. of 7 animals in each group (14 rats in sham). BW_0: body weight before coronary occlusion; BW_3: body weight 3 weeks after the operation; BW_7: body weight 7 weeks after the operation; LV: left ventricle; RV: right ventricule; ND: not detected; MI: myocardial infarction; ENP: enalapril (10 mg/kg/day); LOS: losartan (20 mg/kg/day). *$P < 0.05$ compared with sham group; #$P < 0.05$ compared with MI group.

Table 8. General characteristics of hemodynamic parameters in myocardial infarcted rats with or without enalapril, losartan treatment for 4 weeks starting at 3 weeks after coronary occlusion.

	Sham	Sham + ENP	Sham + LOS	MI	MI + ENP	MI + LOS
Heart rate (beats/min)	231 ± 31	262 ± 9	287 ± 23	250 ± 31	287 ± 17	279 ± 10
LVSP (mm Hg)	121 ± 5.6	113 ± 7.3	107 ± 8.2	112 ± 12	101 ± 2.8	120 ± 11
LVEDP (mm Hg)	4.3 ± 0.4	3.9 ± 0.3	3.8 ± 0.2	18.9 ± 3.1*	7.2 ± 0.6*#	7.5 ± 0.5*#
+dP/dt (mm Hg/sec)	7988 ± 745	6844 ± 566	6683 ± 633	5194 ± 650*	6235 ±318*#	6117 ± 685*#
-dP/dt (mm Hg/sec)	8216 ± 834	7945 ± 722	7490 ± 450	6069 ± 717*	7799 ± 335#	7093 ± 427#
MAP (mm Hg)	104 ± 9	94 ± 9	93 ± 8	101 ± 11	93 ± 8	92 ± 8

Values are mean ± S.E. of 7 animals in each group. LVSP: left ventricular systolic pressure; LVEDP: left ventricular end diastolic pressure; MI: myocardial infarction; MAP: mean arterial pressure; ENP: enalapril (10 mg/kg/day); LOS: losartan (20 mg/kg/day). *$P < 0.05$ compared with sham group; #$P < 0.05$ compared with MI group.

The analysis of EKG (Fig. 2) was focused on the changes of Q waves in Leads I, aVL and -aVR, which are associated with left coronary artery occlusion (Table 9). Not only is the presence of Q wave considered to indicate the existence of MI but also the depth of Q wave indicates the size of the infarcted area (163). Before the operation, there were no obvious Q waves, however, during 7 weeks after the operation, there were large Q waves in the MI groups. No Q wave was observed in sham groups 7 weeks after surgery, although there were some small Q waves during the first week of the operation. The magnitude of Q waves in MI groups were not altered after the treatment with ENP (Table 9).

c. SR Ca^{2+}-regulating protein contents

In order to investigate alterations in SR Ca^{2+}-regulating proteins, Western blots of SR membranes were obtained by employing specific antibodies for RYR, SERCA2, PLB and CQS proteins from sham and infarcted animals with or without ENP therapy (Fig. 3). Densitometric analysis of the immunoblots exhibited a reduction in the relative protein contents for RYR, SERCA2 and PLB in MI rats in comparison to the sham group (Fig. 4 and Fig. 5). Such depressions in these protein contents were partially prevented by the treatment with ENP (Fig. 4 and Fig. 5). In contrast, there was no significant change in the relative value of CQS content in the MI group with or without ENP treatment (Fig. 4). Treatment of sham animals with ENP had no effect on any of the SR Ca^{2+}-regulating proteins (Fig. 4 and Fig. 5).

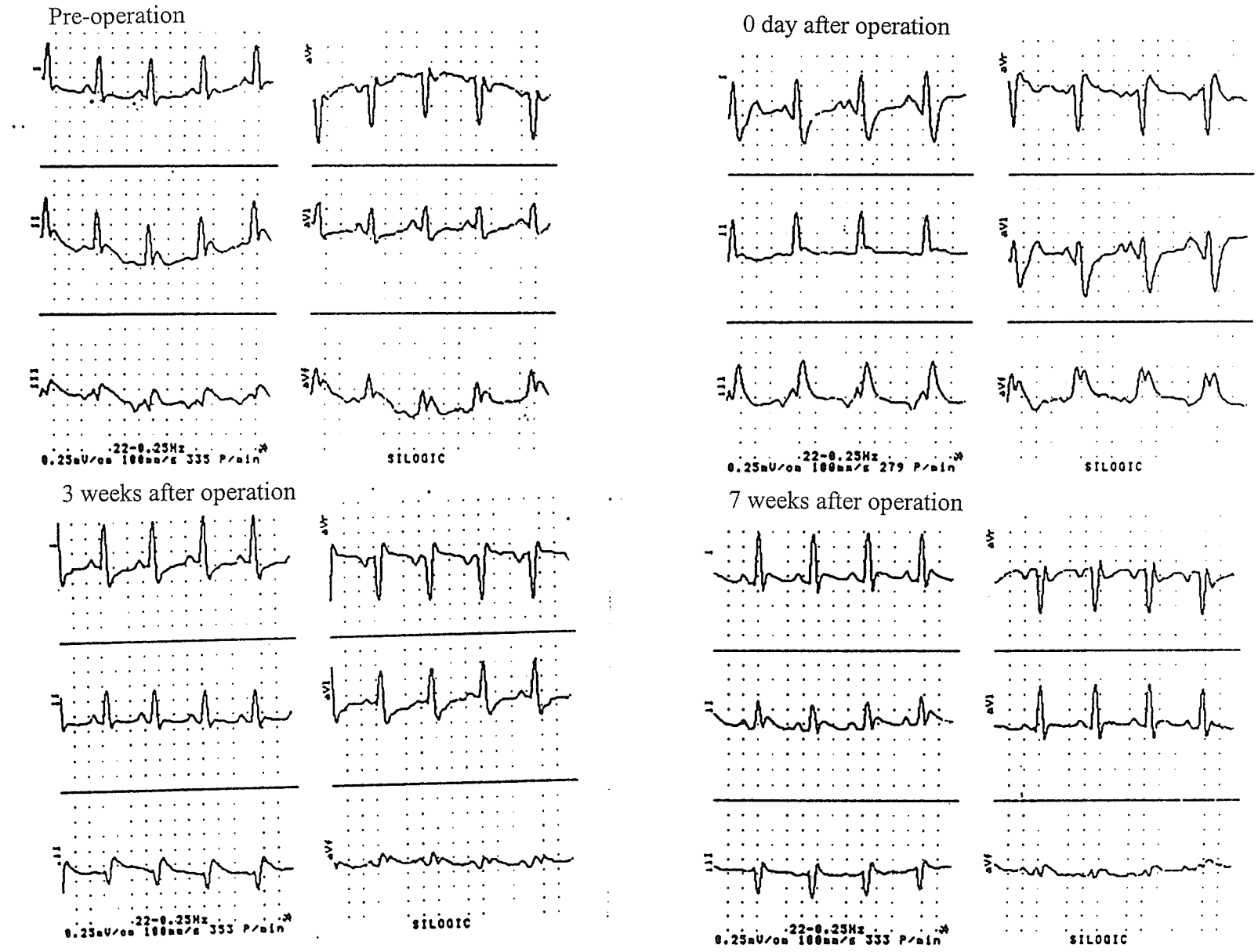

Figure 2. **Typical EKG alteration after MI in rats, from before operation to 7 weeks after coronary ligation.**

Table 9. Electrocardiographic changes (depth of Q wave) of myocardial infarcted rats with or without enalapril, or losartan treatment at 0 days, 3 weeks and 7 weeks after coronary occlusion.

		Sham	Sham + ENP	Sham + LOS	MI	MI + ENP	MI + LOS
0 day	Q_I	0.11 ± 0.02	ND	ND	0.29 ± 0.03*	ND	ND
	Q_{aVL}	0.08 ± 0.01	ND	ND	0.33 ± 0.04*	ND	ND
	Q_{-aVR}	0.15 ± 0.01	ND	ND	0.33 ± 0.03*	ND	ND
3 weeks	Q_I	0.07 ± 0.01	ND	ND	0.75 ± 0.08*	ND	ND
	Q_{aVL}	0.05 ± 0.01	ND	ND	0.82 ± 0.08*	ND	ND
	Q_{-aVR}	0.09 ± 0.01	ND	ND	0.76 ± 0.07*	ND	ND
7 weeks	Q_I	0	0	0.10 ± 0.01	0.84 ± 0.18*	0.63 ± 0.13*	0.66 ± 0.08*
	Q_{aVL}	0	0	0.10 ± 0.01	0.86 ± 0.09*	0.67 ± 0.06*	0.68 ± 0.05*
	Q_{-aVR}	0	0.06 ± 0.01	0.14 ± 0.01	0.52 ± 0.05*	0.46 ± 0.04*	0.41 ± 0.04*

Values are mean ± S.E. There were 24 animals in sham and MI groups for 0 day and 3 week experiments and 8 animals in each group for 7 week experiments. ND: not detected; ENP: enalapril; LOS: losartan; MI: myocardial infarction. *$P < 0.05$ compared with sham group.

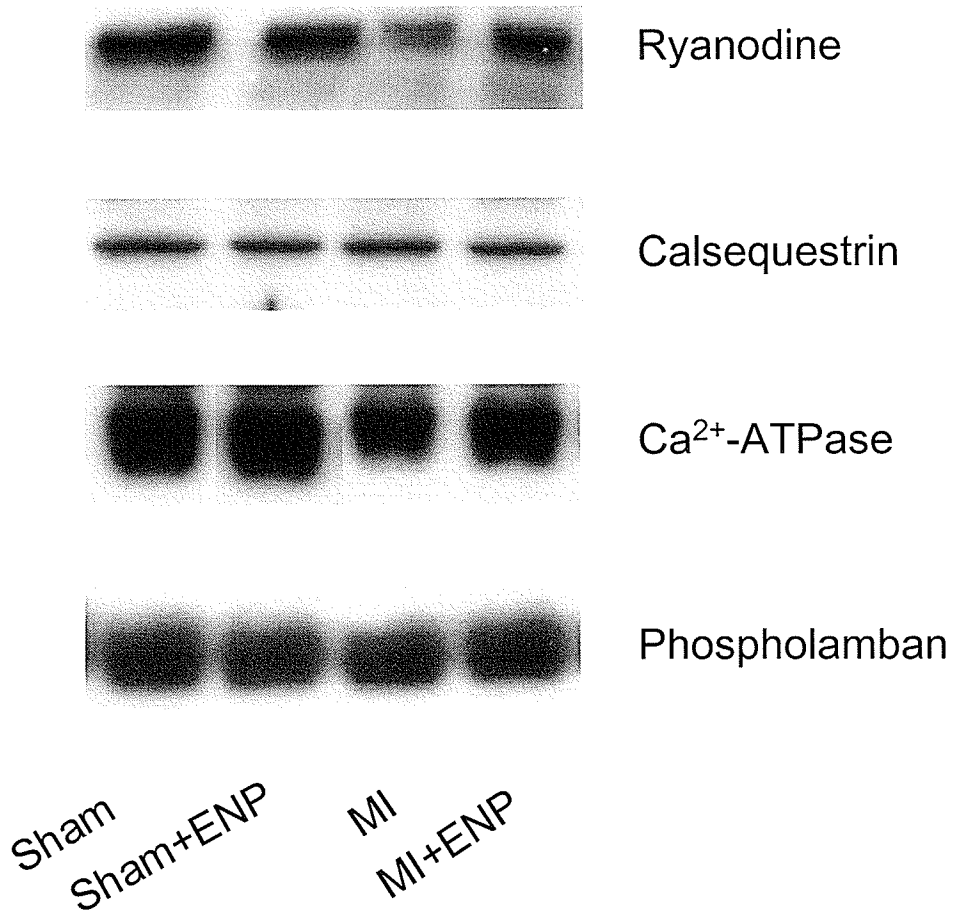

Figure 3. Typical Western blots of SR Ca^{2+}-regulating proteins in LV from sham and 7 weeks infarcted (MI) rats with or without enalapril (ENP) treatment. Immunoblots obtained by using antibodies specific for each protein. ENP was given orally (10mg/kg/day).

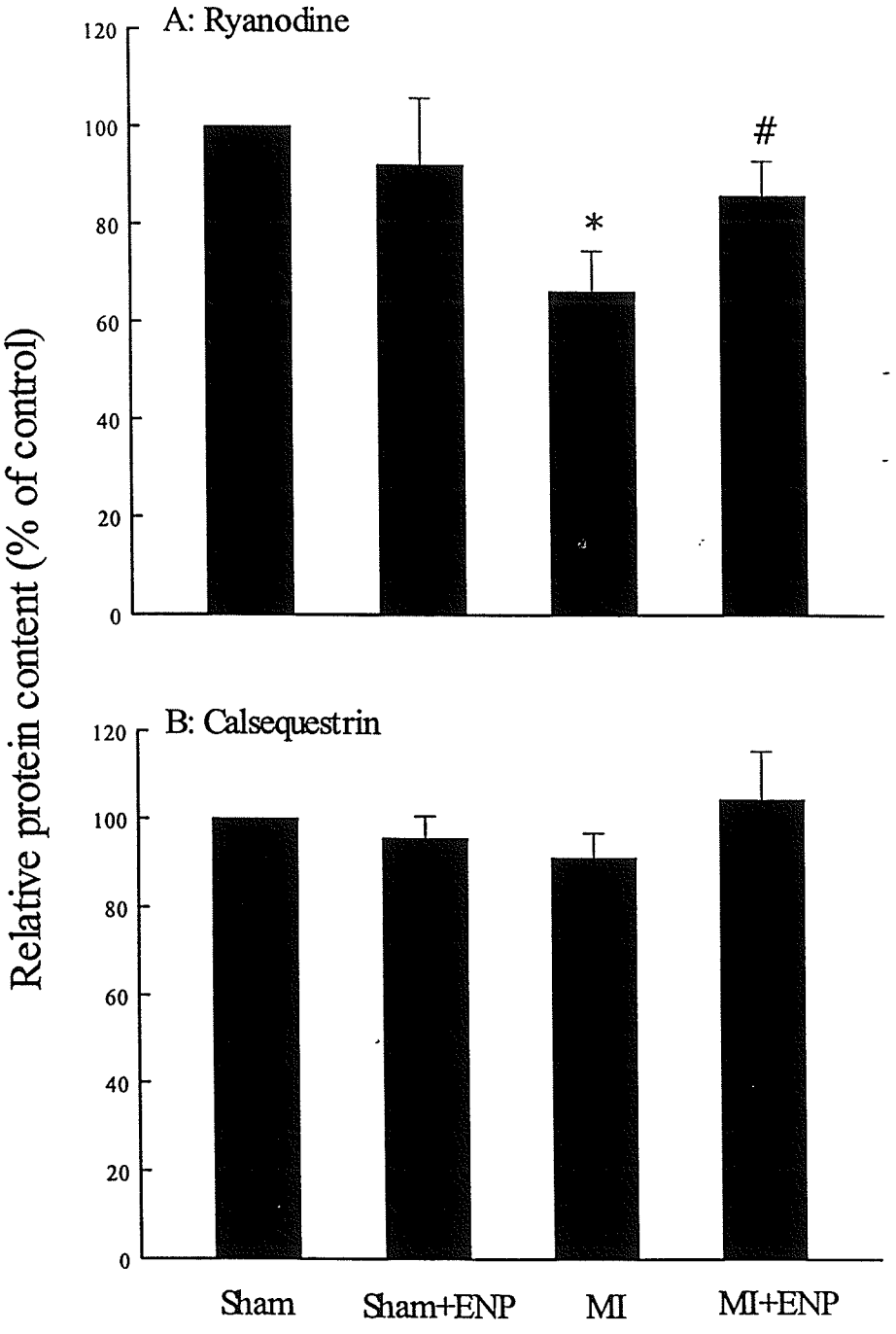

Figure 4. Relative protein content of SR ryanodine (RYR) and calsequestrin (CQS) in LV from sham and 7 weeks infarcted (MI) rats with or without enalapril (ENP) treatment. ENP was given orally (10mg/kg/day). Values are mean ± S.E. of 7 samples in each group. * $P < 0.05$ compared with sham. # $P < 0.05$ compared with MI group.

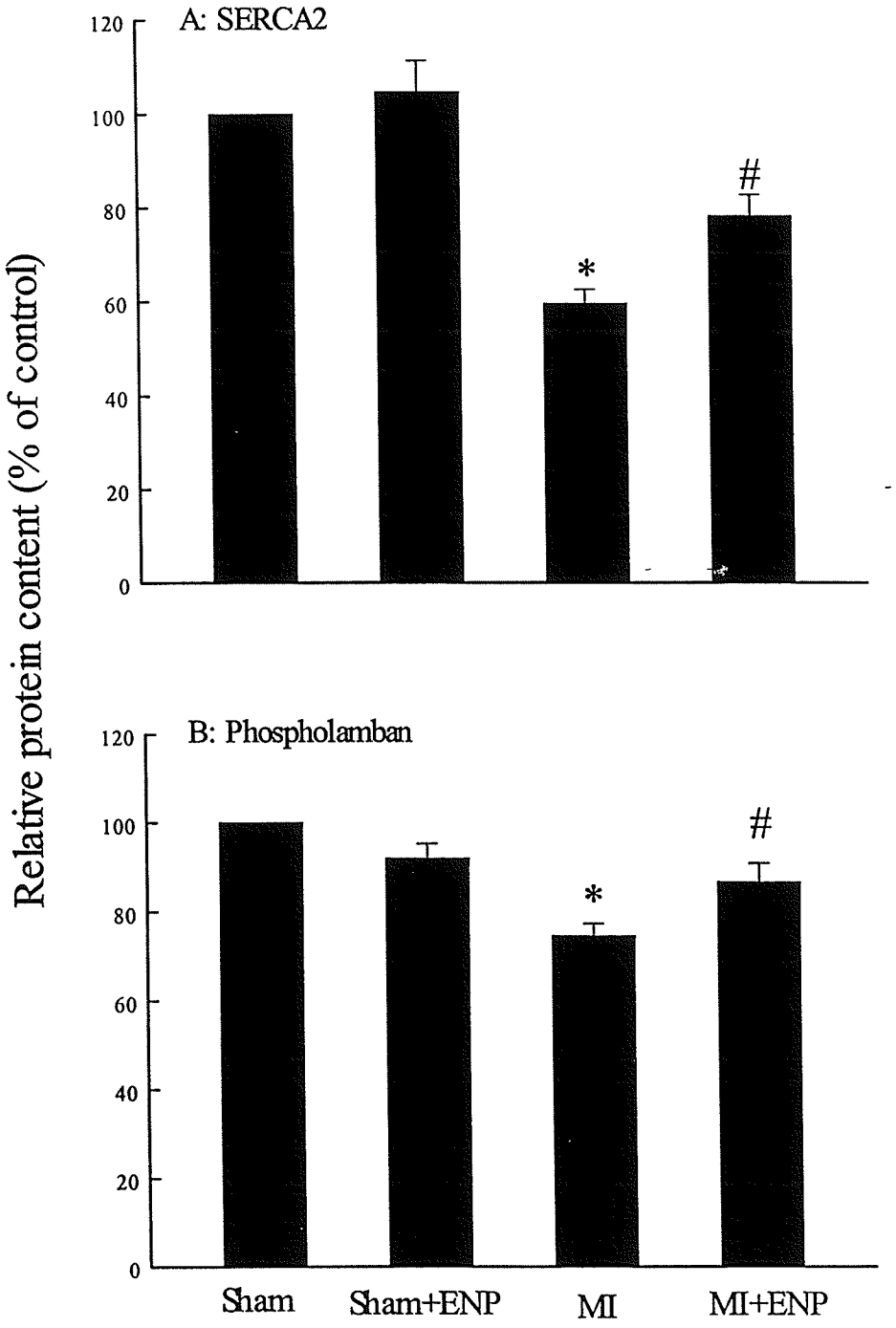

Figure 5 Relative protein content of SR SERCA2 and phospholamban (PLB) in LV from sham and 7 weeks infarcted (MI) rats with or without enalapril (ENP) treatment. ENP was given orally (10mg/kg/day). Values are mean ± S.E. of 7 samples in each group. * $P < 0.05$ compared with sham. # $P < 0.05$ compared with MI group.

d. SR Ca^{2+}-regulating protein gene expression

In order to evaluate the molecular mechanisms for the observed changes in SR Ca^{2+}-regulating proteins in the failing hearts, the mRNA levels specific for these proteins in the viable LV were investigated as well (Figure 6). Northern blots for GAPDH mRNA were used as an internal standard for normalizing the data. The 28S and 18S bands represent the quality of RNA preparations employed in the experiment. A depression of mRNA abundance for RYR, SERCA2 and PLB after MI was observed by the densitometric analysis of the Northern blots. ENP treatment partially prevented the attenuation in mRNA level. However, there was no marked alteration in CQS mRNA level in the MI groups with or without ENP compared to sham value (Fig. 7 and Fig. 8). The mRNA levels for SR RYR, SERCA2, PLB and CQS in the sham animals were not affected by ENP (Fig. 7 and Fig. 8).

2. Effects of LOS on LV and SR After MI

a. General and cardiac parameters, LV Function and EKG changes

In order to test whether the beneficial effects of ENP in the MI groups are mediated through a reduction in RAS activity, the effects of LOS treatment were examined. The data in Table 7 show that increases in the whole heart weight, heart/body weight ratio and lung wet/dry weight ratio in the coronary artery ligated animals were partially attenuated in losartan therapy group (MI + LOS). Losartan therapy had no effects on the scar weight (Table 7). The significant decrease in +dP/dt and -dP/dt and increase in LVEDP in MI rats were partially normalized after the therapy of LOS (Table 8). No significant differences in

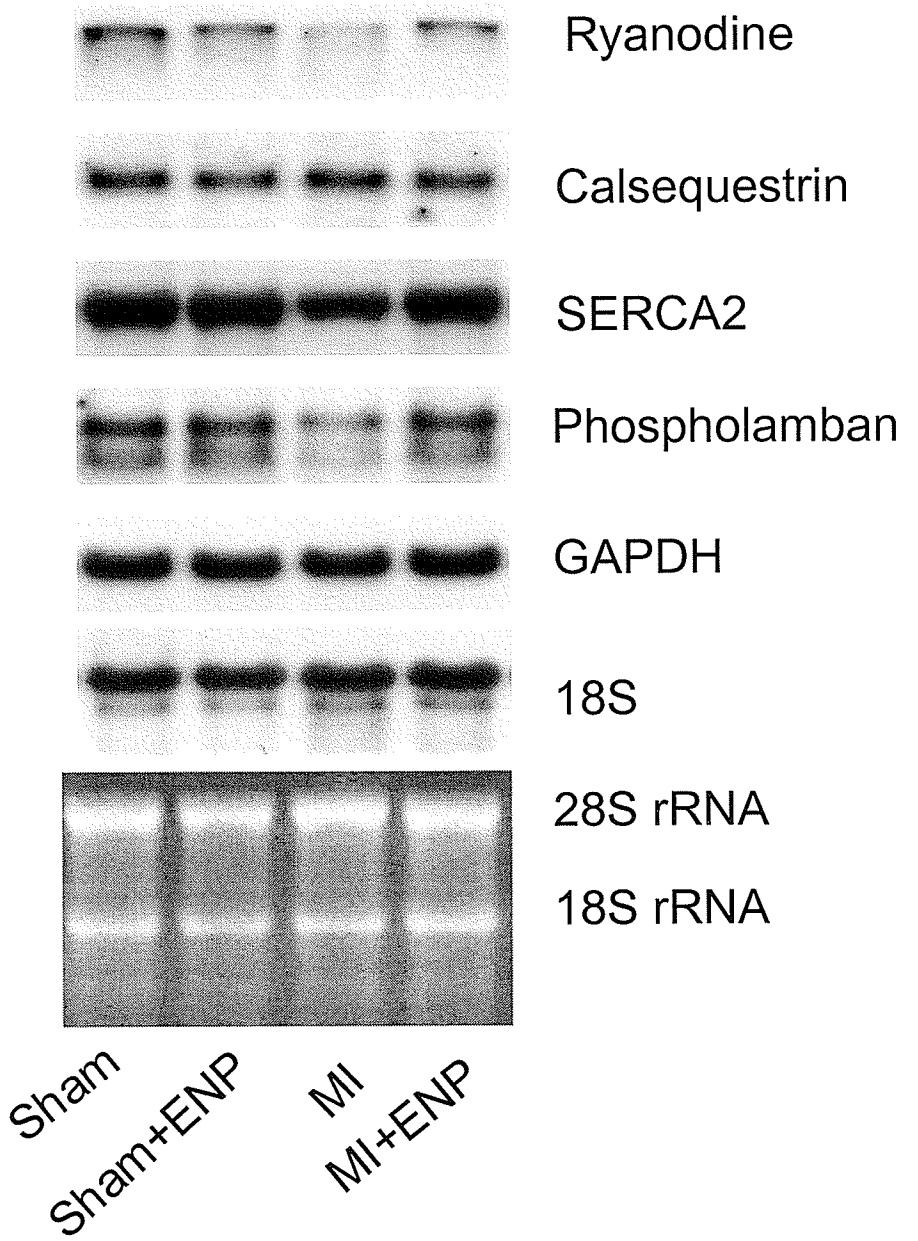

Figure 6. Typical Northern blots of SR Ca^{2+}-regulating proteins in LV from sham and 7 weeks infarcted (MI) rats with or without enalapril (ENP) treatment. ENP was given orally (10mg/kg/day). Blots for ryanodine receptor (RYR), Ca^{2+}-stimulated ATPase (SERCA2), phospholamban (PLB) and calsequestrin (CQS) mRNA were obtained by using specific molecular probes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level was used as internal standard for correcting loading variation in each group. The quality of mRNA preparation is evident from the ethidium bromide staining of the 28S and 18S ribosomal RNA.

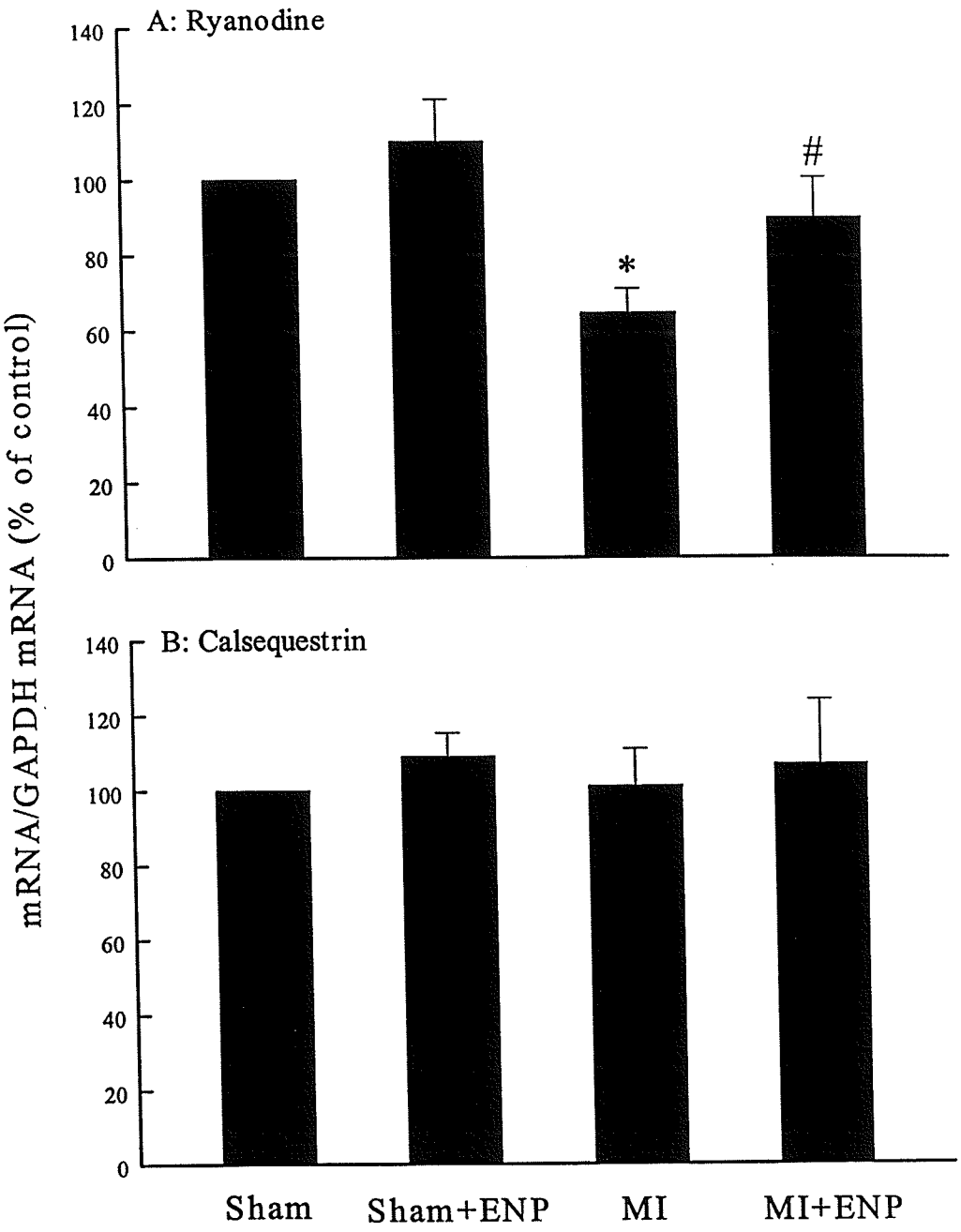

Figure 7. mRNA abundance for SR ryanodine receptor (RYR) and calsequestrin (CQS) in LV from sham and 7 weeks infarcted (MI) rats with or without enalapril (ENP) treatment. ENP was given orally (10mg/kg/day). The values were normalized with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels and are expressed as percentage of sham. Each value is a mean ± S.E. of 6 samples in each group. * $P < 0.05$ compared with sham control. # $P < 0.05$ compared with MI group.

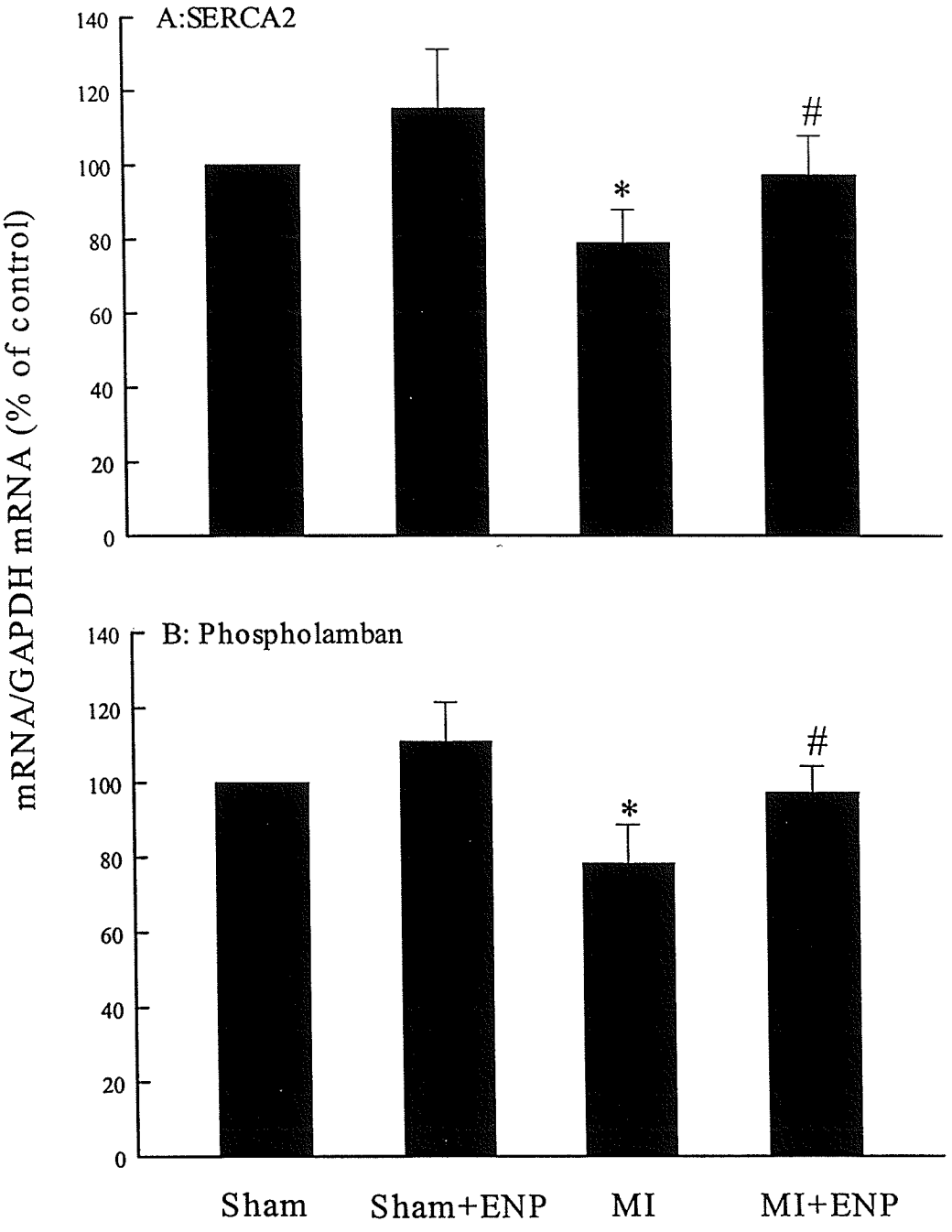

Figure 8. mRNA abundance for SR Ca2+-stimulated ATPase (SERCA2) and phospholamban (PLB) in LV from sham and 7 weeks infarcted (MI) rats with or without enalapril (ENP) treatment. ENP was given orally (10mg/kg/day). The values were normalized with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels and are expressed as percentage of sham. Each value is a mean ± S.E. of 6 samples in each group. * $P < 0.05$ compared with sham control. # $P < 0.05$ compared with MI group.

heart rate, LVSP or MAP were observed in sham or MI groups with or without LOS treatment. The frequency and magnitude of the Q wave in Lead I, aVL and -aVR in the MI group were not altered by treatment with LOS (Fig. 2 and Table 9).

b. SR Ca^{2+}-regulating protein contents and gene expression

Western blots of SR membranes from sham and infarcted animals with or without LOS therapy were also evaluated by using specific antibodies to the corresponding proteins (Figure 9). The relative protein contents for RYR, SERCA2 and PLB were significantly decreased without any changes in CQS content in the MI group in comparison to the sham group; these changes were partially normalized after treatment of LOS (Fig. 10 and Fig. 11). LOS did not affect the Ca^{2+}-regulating proteins in sham group (Fig. 10 and Fig. 11). The mRNA levels specific for the SR Ca^{2+}-regulating proteins including RYR, SERCA2, PLB and CQS in the left ventricles from four groups were investigated (Fig. 12). Densitometric analysis of the Northern blots revealed that depressions in RYR, SERCA and PLB mRNA levels in the MI group were partially prevented by losartan therapy. However, there was no alteration in CQS mRNA level in the failing hearts compared to sham (Fig. 13 and Fig. 14). The mRNA levels for RYR, SERCA2, PLB and CQS in the sham animals were not affected by LOS treatment (Fig. 13 and Fig. 14).

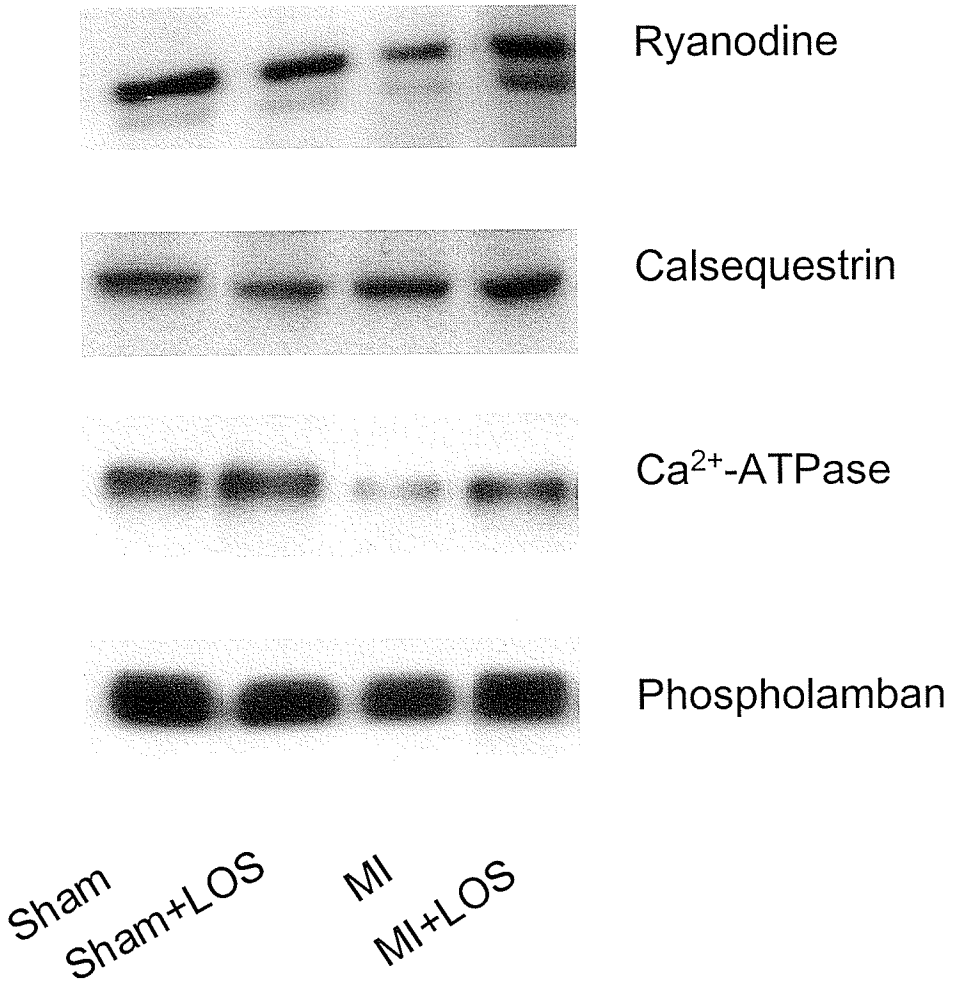

Figure 9. Typical Western blots of SR Ca^{2+}-regulating proteins in LV from sham and 7 weeks infarcted (MI) rats with or without losartan (LOS) treatment. Immunoblots obtained by using antibodies specific for each protein. LOS was given orally (20mg/kg/day).

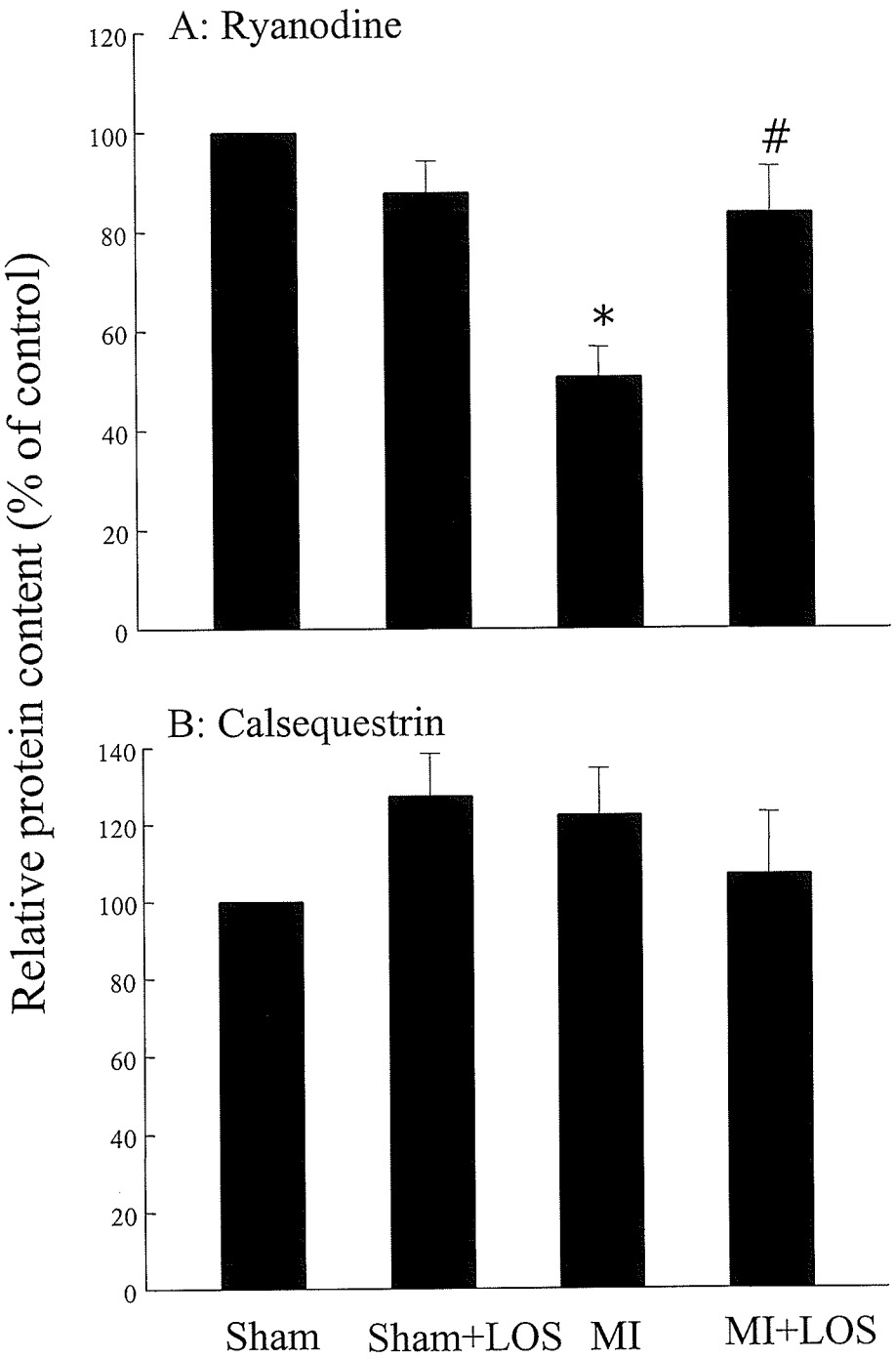

Figure 10. Relative protein content of ryanodine (RYR) and calsequestrin (CQS) in LV from sham and 7 weeks infarcted (MI) rats with or without losartan (LOS) treatment. LOS was given orally (20mg/kg/day). Values are mean ± S.E. of 5 samples in each group. * $P < 0.05$ compared with sham. # $P < 0.05$ compared with MI group.

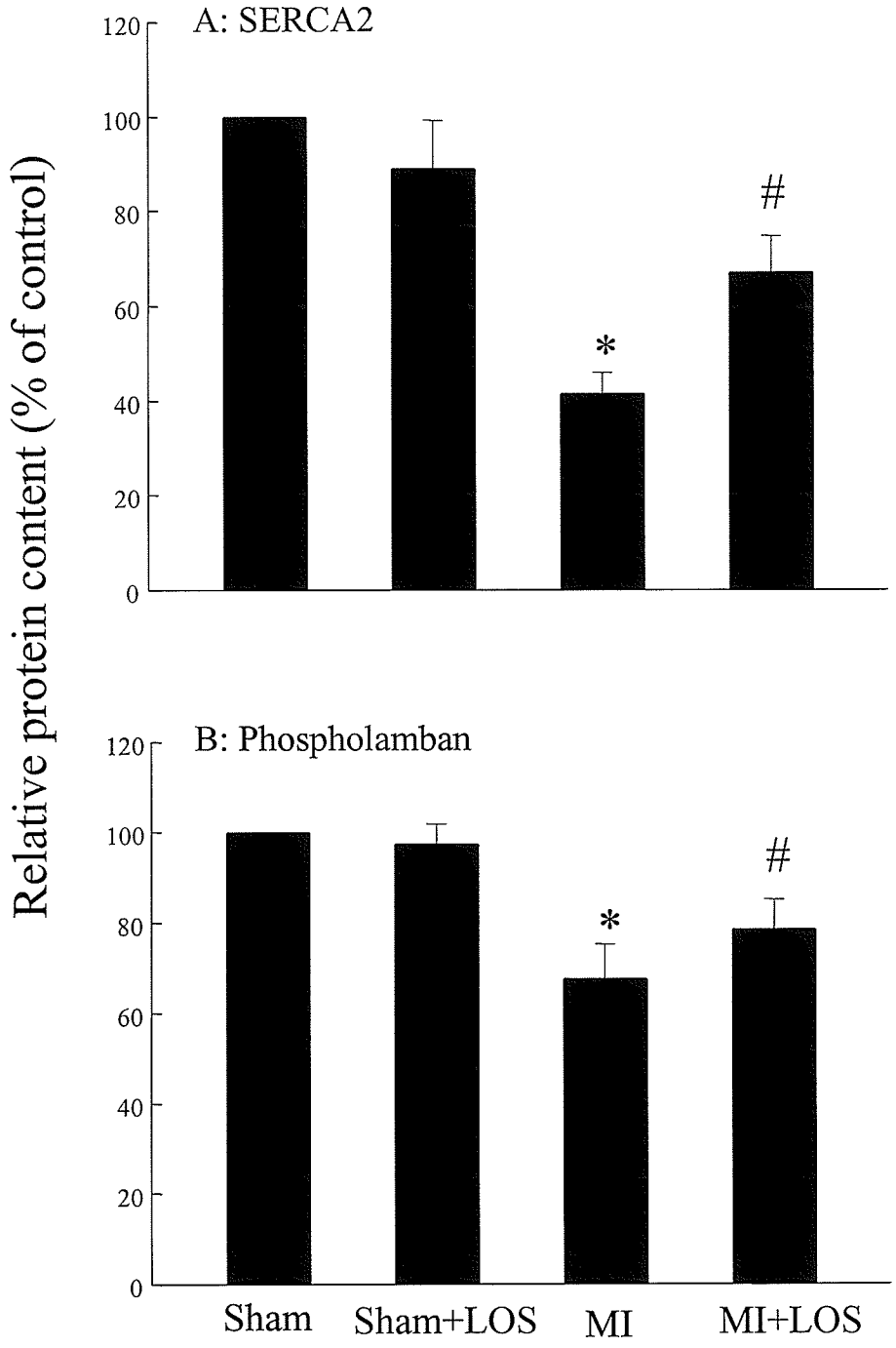

Figure 11. Relative protein content of SERCA2 and phospholamban (PLB) in LV from sham and 7 weeks infarcted (MI) rats with or without losartan (LOS) treatment. LOS was given orally (20mg/kg/day). Values are mean ± S.E. of 5 samples in each group. * $P < 0.05$ compared with sham. # $P < 0.05$ compared with MI group.

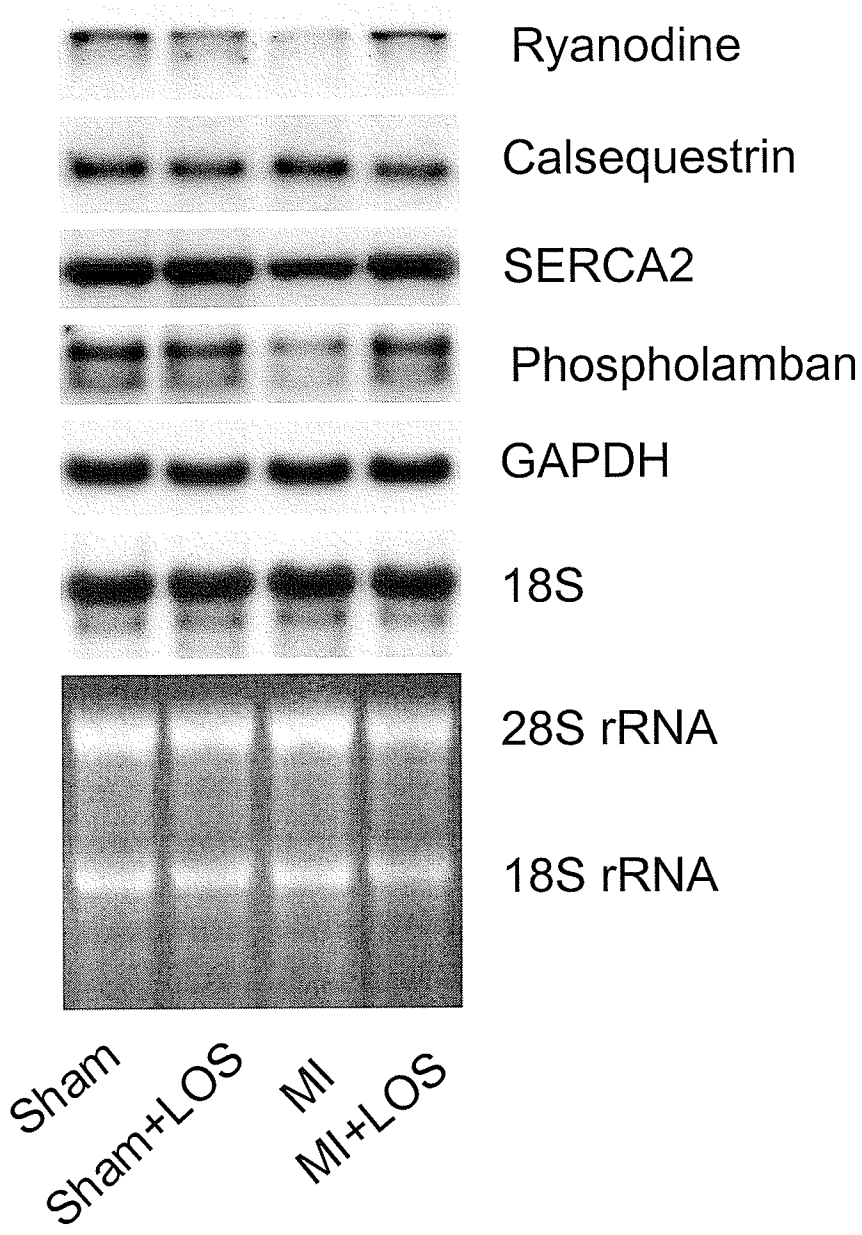

Figure 12. Typical Northern blots of SR Ca^{2+}-regulating proteins in LV from sham and 7 weeks infarcted (MI) rats with or without losartan (LOS) therapy. LOS was given orally (20mg/kg/day). Blots for ryanodine receptor (RYR), Ca^{2+}-stimulated ATPase (SERCA2), phospholamban (PLB) and calsequestrin (CQS) mRNA were obtained by using specific molecular probes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level was used as internal standard for correcting loading variation in each group. The quality of mRNA preparation is evident from the ethidium bromide staining of the 28S and 18S ribosomal RNA.

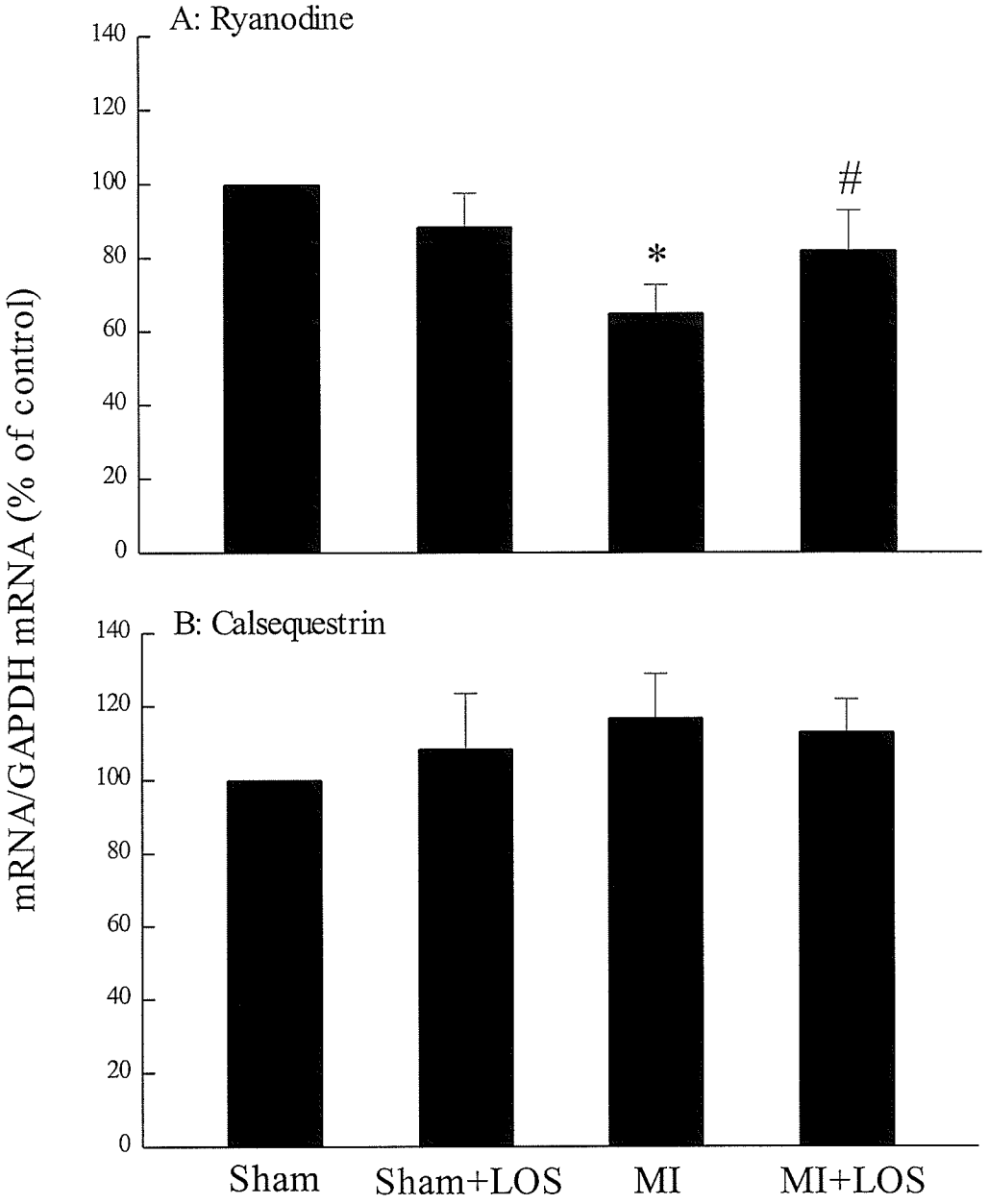

Figure 13. mRNA abundance for SR ryanodine receptor (RYR) and calsequestrin (CQS) in LV from sham and 7 weeks infarcted (MI) rats with or without losartan (LOS) treatment. LOS was given orally (20mg/kg/day). The values were normalized with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels and are expressed as percentage of sham. Each value is a mean ± S.E. of 6 samples in each group. * $P < 0.05$ compared with sham. # $P < 0.05$ compared with MI group.

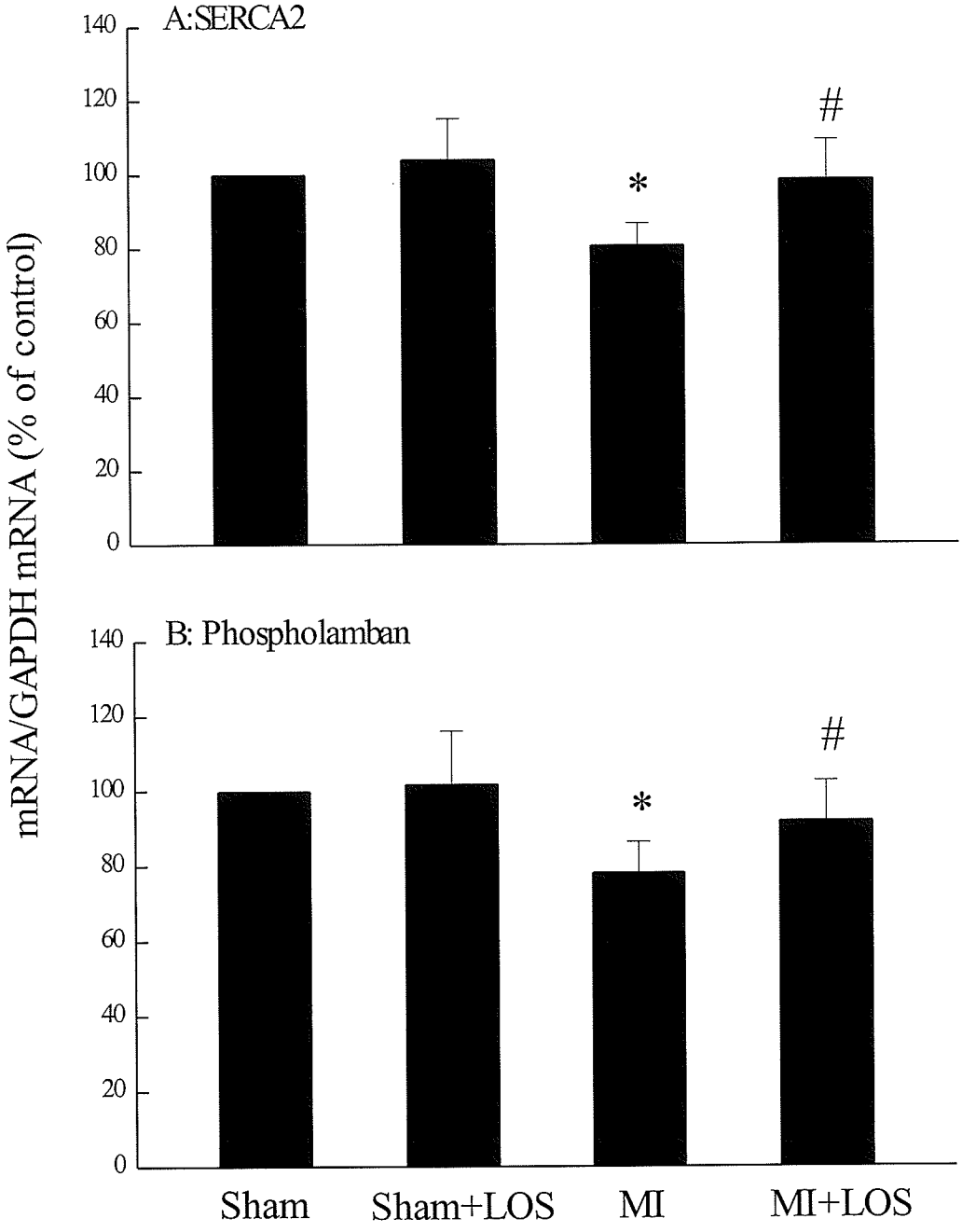

Figure 14. mRNA abundance for SR Ca^{2+}-stimulated ATPase (SERCA2) and phospholamban (PLB) in LV from sham and 7 weeks infarcted (MI) rats with or without losartan (LOS) treatment. LOS was given orally (20mg/kg/day). The values were normalized with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels and are expressed as percentage of sham. Each value is a mean ± S.E. of 6 samples in each group. * $P < 0.05$ compared with sham. # $P < 0.05$ compared with MI group.

V. DISCUSSION

Heart failure has been identified as a major social and economic burden, and it is mostly due to ischemic heart disease, especially after MI (2, 3). After MI, the structural remodeling of the left ventricle is a time-dependent process, which involves complex alterations in ventricular architecture including both the infarcted necrotic heart and noninfarcted viable myocardium (5,6). Heart failure after MI is due to both the loss of contractile tissues and the remodeling of viable tissue. As compensation to maintain stroke volume after the loss of contractile tissue, time-dependent secondary changes in ventricular enlargement and hypertrophy of the viable myocytes take place (5, 6, 8-10). The cellular alterations in cardiomyocytes, which constitute the majority (76%) of the heart, are more important and the remodeling process of myocyte itself may contribute to the development of heart failure (4). Based on previous work in our lab, the Ca^{2+} regulating function of SR is greatly reduced in heart failure induced by coronary ligation in SD rats, which may explain the mechanism of impaired contractility. Many new therapeutic strategies have been applied in clinical practice and get beneficial effects with the development of pharmacology. However, the mechanisms of some therapeutic effects and myocyte remodeling after MI are still not clearly understood. In addition, during the past 2 decades it has been found that ACEIs and Ang II receptor antagonists have very good clinical results in heart failure after MI. The hemodynamic parameters and clinical symptoms are greatly improved after treatment with ACEI, Ang II receptor antagonists or both. However, the therapeutic mechanisms of ACEI and Ang II receptor antagonists in CHF are not fully described.

No animal model has all the salient pathophysiological features desired to mimic the real pathological condition, however, the post-MI rat which was chosen in this project is one of the most accepted models (18, 20-22). In rat, left coronary artery ligation induces sustained and severe MI as the necrotic myocardium becomes completely replaced by scar tissue with 3 to 4 weeks (24); and cardiac failure begins to occur by 4 weeks after the induction of MI (1, 35, 36). In this project, heart failure was confirmed by hemodynamic alterations, including decrease in left ventricular positive and negative dP/dt and a marked rise in left ventricular end-diastolic pressure (LVEDP) in addition to an increase of lung wet/dry weight ratio. The differences of these parameters between sham, MI and MI treatment groups are significant. However, the other changes in heart failure after MI were not significant. Neither left ventricular systolic pressure nor MAP was different between sham and MI group, which may be due to the time point of heart failure development. Seven weeks after operation of coronary artery ligation may not produce severe heart failure but only mild/moderate heart failure which is limited to the left ventricle only. Meanwhile, the liver wet/dry weight ratio did not change among each experimental group, and the abdomen cavity was clear without liquid accumulation. Hepatic edema or ascites was not found when rats were sacrificed, which appears later when the right ventricle is affected (1, 2, 20, 23, 24).

The existence of heart failure in rats is evident from this study, and the left ventricular function is greatly impaired in the ligated animals. Both the systolic (+dP/dt) and diastolic function (-dP/dt and LVEDP) of left ventricle have been affected, and pulmonary congestion

following LV hemodynamic disorder is present as well. The heart failure was well established in this model, which can partially mimic the clinical pathologic condition of heart failure in despite of different etiology and spices. However, the consequences of contractile tissue lost and mechanism of depressed cardiomyocyte contractile function in this model may be same as the heart failure in human being.

Mechanical function was depressed both in the isolated cardiomyocytes of human failing heart and skinned rat cardiomyocytes, (41, 43). At present, it is well accepted that the alteration in cardiomyocyte contractile function may explain the decreased left ventricular function, while left ventricle dysfunction following MI may also be due to an increase in collagen deposition in the viable tissue.

Intracellular Ca^{2+} homeostasis in cardiomyocytes is maintained chiefly by SR. Previous work in our laboratory showed that the Ca^{2+} regulating function of SR is greatly impaired while the left ventricular function was greatly depressed after MI in rats (167). The abnormality of Ca^{2+} transportation in the cell is believed to be the major determinant of myocardial contractility in heart failure after MI. In the process called Ca^{2+}-induced Ca^{2+}-release (73), Ca^{2+} released from (RYR) or absorbed by (SERCA) SR plays an important role in intracellular Ca^{2+} concentration in systole and diastole period, especially in the MI induced heart failure (167). In failing hearts after MI in rats, the diastolic [Ca^{2+}] was increased and systolic peak [Ca^{2+}] was reduced, while the contractile force development and relaxation of heart were reduced (27, 81, 82). Based on our previous data, the SR Ca^{2+} regulating protein activities are reduced after MI while the protein contents of RYR, SERCA and PLB,

associated with decreased cardiomyocytes contractility and LV hemodynamic function. These changes are confirmed in the present series of experiments as well.

The SR Ca^{2+} regulating protein RYR, SERCA, PLB and CQS control the Ca^{2+}-release, -uptake and -storage activities. Depression of cardiac pump function, a hallmark of heart failure, has been associated with a reduction in cardiac SR Ca^{2+}-transport. The disability of RYR impairs the release of Ca^{2+} from SR, while SERCA and PLB affect the Ca^{2+} sequestration from cytosol. In our study both the protein contents and mRNA level were altered after MI in rats. There are significant differences of protein contents between sham and MI groups, which may explain the depressed contractility of left ventricle due to the depressed SR function. Meanwhile, the mRNA level of RYR, SERCA and PLB are decreased as well in the MI groups compared with sham, which indicates that the mRNA level of Ca^{2+} regulation protein was positively correlated with protein contents and cardiac function. These data agree with our previous studies and these alterations in RYR, SERCA and PLB after MI, can be attenuated by treatment with ACEIs, such as captopril (167). Some reports showed that the SERCA2 mRNA and protein levels were not decreased in the MI rats and this may be due to the lack of presence of CHF and the relatively small average MI size (29%) in the LV (34, 166).

In our experiments, the effect of ENP (ACEI) and LOS (Ang II receptor antagonist) on SR function were originally evaluated. And it was found that after the 4 weeks therapy of ENP (10 mg/kg/day) or LOS (20 mg/kg/day), the general characteristics and hemodynamic changes of MI rats were partially prevented. Since neither the scar weight nor the frequency

or magnitude of the Q wave in the EKG recording was altered upon treatment of the MI animal with ENP or LOS, it is unlikely that the observed hemodynamic alteration by ENP or LOS were due to changes in the scar size. These data are consistent with the existence of MI induced CHF in rats (40, 165) and the therapeutic effects of ENP on cardiac performance in this heart failure model.

Since ENP decreases both the plasma and myocardial levels of Ang II in the MI animals, it is likely that the beneficial effects of ENP are due to its inhibitory actions on both the tissue and circulatory ACE. The action of ACEI on cardiomyocytes is not clear (6), but it may include blockade of cell growth effects of Ang II and potential direct cellular effects of ACE (a carboxypeptidase) (136). The ENP dose in our project did not produce the significant decrease of MAP, which is one of the major effects of ENP. However, the contractility of LV is great improved after treatment. ENP is confirmed to have the hemodynamic benefit direct through cardiomyocyte effects without indirect effect of decreasing afterload or changing blood pressure.

The treatment began at 3 weeks after MI, when the scar was completely healed and heart failure is progressing due to the remodeling of viable cardiomyocytes. The peripheral resistance has relatively less effect on cardiomyocytes. This method is well used for the chronic heart failure model, which may decrease the peripheral blood pressure effect of ENP with the establishment development of CHF. Therefore ENP was utilized to evaluate its therapeutic effects mainly on the cardiomyocytes, instead of blood vessels. Coronary artery ligated animals were divided into MI model until 3 weeks after operation, or CHF model

from 3 week after operation (1, 167).

Our study is the first examination of the direct effect of ENP on SR Ca^{2+} regulating proteins, RYR, SERCA and PLB in MI induced heart failure model. The decrease of protein contents may be due to enhanced degeneration of protein, or decreased production of protein during the development of heart failure. Meanwhile, the downregulation of mRNA of each protein was observed at 7 weeks after operation. ENP can attenuate these Ca^{2+} regulating proteins both in protein and mRNA levels. This may explain the possible mechanism of ENP via altering the mRNA production or degeneration and relatively increasing the protein level. There may be increased protein degeneration, however, the impaired mRNA level could be the major reason for the protein reduction in CHF.

Similar results have been found in the LOS (20 mg/kg/day) treatment group. From the general characteristics, hemodynamic alteration, SR Ca^{2+} regulating protein contents to mRNA level of these proteins, LOS partially attenuated the changes in CHF. LOS blocks the AT_1 receptor, increasing the mRNA and protein contents in CHF, and then improves the contractility of cardiomyocytes and left ventricular function. This dose produces little change in peripheral resistance (MAP). The therapy was also begun at 3 weeks after operation when the scar already healed and heart failure was well established, therefore LOS also has a direct effect on the cardiomyocytes, instead of indirect hemodynamic effect via afterload reduction.

In the present study, both ENP and LOS only partially attenuate the RYR, SERCA and PLB changes. This may be explained as followings. RAS has been found important in

heart failure, however, upregulation of RAS itself is not sufficient to produce heart failure after MI. There are many other neural hormonal factors playing roles in CHF, such as adrenergic nervous system, endothelin, vasopressin. Just as in this project, ENP (10 mg/kg/day) or LOS (20 mg/kg/day) can only partially attenuate the alteration in mRNA and protein level, LV contractility and pulmonary edema condition. Second, the dose of treatment may be not enough to produce a hypotensive effect, which is usually found in the patients with ACEI therapy. However, such hypotension effect is usually transient in human and this pressor reducing effect may be different between rats and humans. Third, there may be alternative metabolic pathways for Ang II production; ENP alone may not inhibit all the biologically active RAS. It is well accepted that Ang II can be produced via the local tissue chymase pathway, which is completely independent of ACE and can't be inhibited by ENP. However, the amount of Ang II produced via the local metabolic pathway can not be measured at the present time. It is too early to conclude that complete inhibition of Ang II formation can have more benefit for the treatment of heart failure. In addition, ENP also depletes the inactivation effect of ACE on bradykinin, which has its own biological effects outside of Ang II receptors. The detailed function of bradykinin in heart failure is not clear yet. Finally, Ang II not only activates AT_1 receptor but also AT_2 receptor, which are not blocked by LOS. Also, LOS has no effect on Ang II production, and may even increase Ang II concentration via the negative feedback. Since LOS is reported to have more potential in clinical practice, it was expected the LOS may recover RYR, SERCA or PLB mRNA level in higher degree. However, the results did not show different between ENP and LOS

treatment groups.

Our study provides evidence that ENP and LOS play an important role in modification of SR Ca^{2+} regulation in failing rat heart after MI. Moreover, the Ang I, ACE, Ang II, Ang II receptor and intracellular effectors are believed to be the major metabolic pathway that is interrupted when there is a great change in cardiac function. Furthermore, the cardiac Ca^{2+} regulation protein formation and/or function may be primarily controlled under RAS from 4 to 7 weeks after ligation. At a later time, heart failure may progress partially or completely independently of the neurohormonal status.

In summary, our study is the first evaluation of the effects of ENP and LOS treatment on SR Ca^{2+} regulating proteins in failing hearts after MI. ENP and LOS interface RAS and they play a major role in the Ca^{2+} regulation in heart failure after MI. The mechanism of the therapeutic effects of ENP and LOS in CHF may partly be due to attenuation in SR remodeling in the infarcted heart, particularly the changes of mRNA after MI, which is part of the remodeling of MI heart.

All the biological factors must be via certain intracellular signal pathway to perform their function on cardiomyocytes. RAS and related factors contact with their receptors on SL, activate or inhibit certain signal transduction pathway, which may affect gene expression and protein synthesis. It is indicated in this study that Ang II via Ang II receptor has direct or indirect effect on cardiac gene expression of SR Ca^{2+} regulating proteins. The steps from receptor to intracellular effectors should be the major targets in future research.

VI. CONCLUSIONS

In this study, the therapeutic effects of ENP and LOS on cardiac performance, SR membranes, Ca^{2+}-handling proteins and gene expression in CHF subsequent to MI were examined. On the basis of results obtained above, the following conclusions are drawn:

1. CHF was evident at 7 weeks after ligation of the left coronary artery.
2. The EKG changes at 3 and 7 weeks after operation can be used to test the existence of scar.
3. Remodeling of SR membrane in failing heart is associated with alteration in RYR, SERCA and PLB protein levels and are due to changes in cardiac gene expression for specific proteins.
4. ENP and LOS significantly improved the cardiac function and clinical signs of CHF following MI without changes in infarct weight or Q wave.
5. The beneficial effects of ENP and LOS therapy on cardiac function were related to the prevention of remodeling of SR membrane Ca^{2+}-handling proteins, by modification of gene expression specific for SR proteins.
6. The beneficial effects of ACE1 in heart failure due to MI may be due to a reduction in the RAS activity.

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