Bull Yamaguchi Med Sch 43(3-4) : 1996

Effects of Ischemic Preconditioning on Ventricular Function and Ryanodine Receptors of Cardiac Sarcoplasmic Reticulum in Dogs

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(Received October 1, 1996, revised October 31, 1996)

Abstract Previous studies have shown that myocardial ischemia causes a reduction in the number of cardiac ryanodine receptors. However, how ischemic preconditioning(IP) affects the density of ryanodine receptors remains uncertain. To clarify the effect of IP on ryanodine receptors, the density of high-affinity ^{3}H-ryanodine binding sites and the dissociation constant for ryanodine of the myocardium were examined in anesthetized open chest dogs with or without IP: Group S(n=6); 5 minute-ischemia was followed by 10 minute-reperfusion as IP. Group C(n=5); 20 minute-ischemia was performed without IP. Group P(n=6); 20 minute-ischemia was performed following IP. Myocardium from risk and non-risk areas were obtained for the subsequent purification of sarcoplasmic reticulum microsomal fraction. Hemodynamic parameters showed no significant differences between group C and group P after 20-minute ischemia. However, density of ryanodine receptors in ischemic region was significantly reduced in group C compared to that in non-ischemic region (7.3±0.7 versus 11.3±1.4 pmol/mg protein; $p<0.05$). When preconditioned, the reduction of density of ryanodine receptors in ischemic region was significantly attenuated (11.6±1.4 versus 12.7±0.9 pmol/mg protein). Just after IP procedure, density of ryanodine receptors in the ischemic region did not change significantly compared to the non-ischemic region (10.3±0.5 versus 9.8±0.5 pmol/mg protein). Thus, IP attenuated the down regulation of ryanodine receptors after sustained ischemia, which should play an important role in the recovery of myocardial function after reperfusion.

Key words: Dogs, Ischemic preconditioning, Ryanodine receptors, Sarcoplasmic reticulum

Introduction

Previous works [1-5] have shown that brief periods of ischemic insults brought protective effects for myocardial injuries during subsequent sustained ischemia. This phenomenon was known as ischemic preconditioning (IP) [1]. IP is reported to be protective for the heart by not only narrowing infarct size, but also reducing reperfusion injuries. A number of reports indicated the underlying mechanisms of IP, but its mechanism is still unclear because they vary so wide and might differ among species [6-12].

One of the crucial problems in the pathogenesis during ischemia and reperfusion is cytosolic calcium overload[13-17]. During ischemia and reperfusion calcium overload occurs by the release of calcium from intracellular storage sites and influx of extra cellular

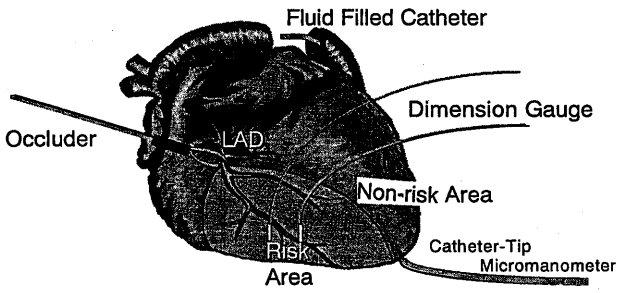

Fig. 1 Experimental model is illustrated: A catheter-tip micromanometer was inserted through the apex stab. The left anterior descending artery was dissected free distal to the first diagonal branch and an occluder was placed around it. A pair of ultrasonic crystals were implanted in the anterior wall.

calcium due to enhanced Na^+-Ca^{2+} exchange. Cell death can be induced in cell suspensions by increasing cytosolic calcium concentration with calcium ionophores[18]. A likely mediator of this cytosolic calcium handling is the sarcoplasmic reticulum (SR) which plays a key role in cardiac muscle contraction and relaxation by rapidly releasing and re-sequestering calcium, at least under the physiological condition [19,20]. Stunned myocardium which indicates the depressed myocardial contraction after ischemia/reperfusion was reported to be concerned deeply with SR function[21,22]. Recently, Zucchi et al.[12] showed brief periods of ischemic insults as IP reduced the density of ryanodine receptors. They indicated that this down regulation of ryanodine receptors might be a mechanism of cardioprotection of IP, likely by attenuating Ca^{2+} overload in rat hearts. To determine whether this down regulation of ryanodine receptors-mediated Ca^{2+} handling is a key role of cardioprotective effect of IP in other species than rats, we studied the effects of IP on ryanodine receptors just after IP and after 20-minute subsequent ischemia in dogs.

Materials and Methods

The protocol of the present study was approved by the animal research committee of this institute. Eighteen dogs (weight; 8-15 Kg, mean 11±2 Kg) were anesthetized with ketamine hydrochloride (2 mg/Kg, subcutaneously), and maintained with an intravenous injection of α-chloralose (50 mg/kg bolus followed by 0.4 mg/kg/min). The dogs were intubated intratracheally and ventilated with positive pressure using a mixture of 100% O_2 and room air with a Harvard respirator. Arterial blood gases were measured during the experiment and pO_2 , pCO_2 and pH were maintained within physiological ranges. A left lateral thoracotomy was performed through the fifth intercostal space. The heart was suspended on a pericardial cradle. The body temperature was maintained at 37± 0.5 ℃ using a heating pad. The experimental design is illustrated in Fig. 1. Aortic pressure was measured with a fluid filled catheter cannulated into the femoral artery. A catheter -tip micromanometer (Millar Instrument INC, TX, USA) was inserted through the apex stab wound into the left ventricle to measure left ventricular pressure (LVP). A zero shift of the pressure was corrected by a simultaneous fluid filled pressure recording (Gould Statham P23Db). The proximal portion of the left anterior descending coronary artery was dissected free and a thread occluder was placed around it. To evaluate the regional contractile function at risk area during ischemia, a pair of ultrasonic crystals were implanted in the left ventricular mid- wall where the blood was supplied by the occluded artery. The change of segmental wall length was continuously measured by the ultrasonic transit time technique[23] (Triton Technologies, San Diego, California, USA). The percent systolic shortening (%SS) was calculated according to the previous report[23] as follows; $\%SS=100\times(EDL-ESL)/EDL$ where EDL= end-diastolic length and ESL=end-systolic length.

The hemodynamic data were all digitized at 3 msec interval using a Codas data analyzing system (Data Q. Instruments, Inc., Ohio, USA). Ten beats were averaged and hemodynamic parameters were calculated.

Protocols: The protocol was shown in Fig 2. The animals were divided into 3 groups. In the control group, after the baseline measurement of hemodynamics, the left anterior descending coronary artery was occluded for

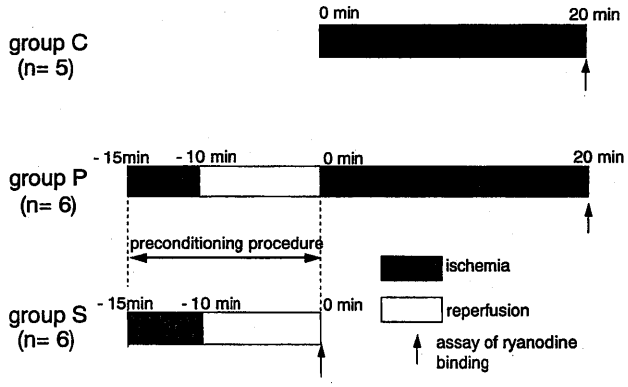

Fig. 2 Protocols are shown: group C indicates control group undergoing a single 20 -minute ischemia, group P indicates preconditioned group undergoing 5 -minute ischemia /10- minute reperfusion as ischemic preconditioning, followed by 20-minute subsequent ischemia, group S indicates preconditioned group without 20-minute subsequent ischemia.

20 minutes and then the heart was rapidly excised and the myocardium was sampled for the subsequent analysis (group C). In the ischemic preconditioned group (group P), a single 5-minute ischemia followed by 10-minute reperfusion was performed before the subsequent 20-minute sustained coronary occlusion and then sacrificed. In the third group, only 5-minute ischemia followed by 10- minute reperfusion was performed and then sacrificed (group S).

Purification of SR; Microsomal fractions enriched in SR vesicles were obtained following the method by Kranias et al[24]. Briefly, after sacrifice using overdose of anesthetics, the hearts were excised immediately and placed in iced saline. A pair of specimens (4-6 g) were obtained from ischemic area and non-ischemic area. Specimens were weighed, minced and homogenized in 4 volumes of 0.3 M sucrose, 30 mM Tris-maleate, 5 mg/L leupeptin, 0.1 mM PMSF, pH 7.4 (solution I) with a Brinkmamm Polytron. The homogenate was centrifuged at 5,500 g for 10 minutes in a centrifuge (KUBOTA 1900, Tokyo). The supernatant filtered by four layers of cheese cloth was centrifuged for 20 minutes at 12,000 g and again centrifuged at 143,000 g in Optima TM TL ultra centrifuge (BECKMAN, CA, USA) for 30 minutes. The pellet was resuspended in a glass homogenizer with 0.3 M sucrose, 30 mM Tris-maleate, 0.6 M KCL, 5 mg/L leupeptin, 0.1 mM PMSF, pH 7.4 (solution II). This suspension was centrifuged at 143,000 g for 45 minutes and the pellet was resuspended with solution II. This suspension was centrifuged at 143,000 g for 45 minutes, and the pellet was resuspended with solution I. This suspension was again centrifuged at 143,000 g for 45 minutes. This pellet was resuspended with 0.3 M sucrose, 20 mM Tris-maleate, 0.1 M KCl, 5 mg/L leupeptin, 0.1 mM PMSF, pH 7.4 (solution III) at a concentration of about 5 mg protein/mL. The resulting suspension enriched in SR vesicles, was frozen and stored at −80 ℃. All centrifugations were performed at 4 ℃. The protein content was determined by the Lowry method[25], using bovine serum albumin as a standard.

Ryanodine binding assay; High-affinity ryanodine binding was determined as described by Zucchi et al.[26] with minor changes. The suspensions enriched in SR vesicles (the protein concentration; 0.1 mg/mL) were incubated at 25 ℃ for 120 minutes in a buffered medium (final volume 1 mL) containing 25 mM imidazole-HCl, 1 M KCl, 0.6 to 20 nM ^{3}H-ryanodine, 0.95 mM EGTA, and 1.103 mM $CaCl_2$, pH 7.4. In some experiments, $CaCl_2$ concentrations were varied to obtain free Ca^{2+} concentrations ranging from 1×10^{-7} to 5×10^{-3} M. The binding reaction was terminated by filtration through filters with pores of 0.45 μm (Millipore Filter, HA, Millipore LTD. Yonezawa, JPN). The filters were washed twice with 5 mL buffered medium used during the incubation and placed in 5 mL scintillation fluid. Radioactivity was measured by liquid scintillation counter (TRI-CARB 4640, PACKARD, Powners Grove, IL). Each vial was counted for a minute. Incubation was performed in duplicate, and non specific binding was determined in the presence of 2.0 μM cold ryanodine. The difference between the counts of duplicate samples was $<10\%$ in all cases. The maximal ryanodine binding to the receptor (Bmax) and the affinity of Ca^{2+} releasing channel to ryanodine (Kd) were obtained by a scatchard plot as previously described[26].

Statistics; Values are expressed as mean± SE. Statistics for hemodynamics, Bmax and Kd of ryanodine receptors among the groups was performed by one way ANOVA followed by Scheffe's multiple comparison test. A p value less than 0.05 was considered significant.

Results

Hemodynamic parameters and regional contractile function before and after 20 minutes ischemia are summarized in Table 1. There was no significant difference in HR, mean aortic pressure, left ventricular end-diastolic pressure, left ventricular (+) and (−) dP/dt as well as regional contractile function expressed as %SS between group C and group P. In group P, hemodynamic variables and regional contractile function returned to the baseline after 5-minute ischemia/10- minute reperfusion. During 5-minute ischemia, %SS was less than zero, indicating dyskinesis of the ischemic wall. LV EDP was significantly increased after 20 minutes ischemia when compared to pre-ischemia. LV (+) and (−) dP/dt and %SS were significantly decreased after 20 minutes ischemia when compared to pre-ischemia.

The recovery of SR from the area at risk was 78±12, 80±11 and 76±9 mg/g, and 76± 13 mg/g, 75±14 mg/g and 71±15 mg/g in groups C, P and S, respectively and no significant difference was detected among the groups. Examples of ^{3}H-ryanodine binding curves obtained at pCa 4.7 are shown in Fig. 3. Nonspecific binding was <10% of total binding at≦2.5 nM ^{3}H-ryanodine. In each case, data were best interpolated by a single -binding site, and linear regression was well performed on the amount of ^{3}H- ryanodine bound versus bound/ free ligand. The mean values of the maximal ryanodine binding to the receptor (Bmax) and the affinity of Ca^{2+} releasing channel to ryanodine (Kd) are shown in Table 2. In group C, Bmax of area at risk was significantly decreased compared to that of area at non risk (7.3±0.6 for ischemic area and 11.3±1.4 pmol/mg protein for non-ischemic area; $p<0.05$). In group P, the decrease of Bmax of area at risk was significantly attenuated compared to that of group C. There was no significant difference in Bmax between the preconditioned area and non -preconditioned area in group S. There is no significant difference in Kd between areas at

Table 1. Hemodynamic data and regional myocardial fuuction

Parameter	Group	N	Pre-ischemia	20-min after ischemia
HR (bpm)	C	5	149±4	150±5
	P	6	142±2	149±3
AOm (mmHg)	C	5	108±8	101±5
	P	6	102±3	97±11
EDP (mmHg)	C	5	8±2	11±2*
	P	6	9±2	12±2*
+dP/dt	C	5	2399±97	2081±113*
	P	6	2395±81	2170±140*
-dP/dt	C	5	−2427±172	−2104±173*
	P	6	−2355±111	−2063±123*
%SS (%)	C	5	22.1±3.2	−7.3±2.5*
	P	6	20.9±3.1	−11.0±1.6*

N, number of canines; C, control group undergoing a single 20 minutes ischemia.; P, preconditioned group followed by 20 minutes ischemia; HR, heart rate; AOm, aortic mean pressure; EDP, left ventricular end diastolic pressurp; dP/dt, the first derivative of the left ventricular pressure; %SS, percent segmental shortening. Values are mean ±SE. *P<0.05 vs pre-ischemia.

Table 2. High-Affinity ^{3}H-Ryanodine Binding to SR Fraction

	area at risk		area at non risk	
	Bmax (pmol/mg)	Kd (nmol/L)	Bmax (pmol/mg)	Kd (nmol/L)
group C	7.3±0.6	7.5±0.9	11.3±1.4#	6.4±0.9
group P	11.6±1.4#	6.7±1.4	12.7±0.9#	7.2±0.5
group S	10.3±0.5#	5.0±0.8	9.9±0.5#	5.1±0.8

SR, sarcoplasmic reticulum; group C(N=5), control group undergoing a single 20-minute ischemia; group P(N=6), preconditioned group followed by 20-minute ischemia; group S(N=6), preconditioned group without 20-minute ischemia. Bmax, number of binding sites; Kd, dissociation constant. Values are mean ±SE. # P<0.05 vs. area at risk in group C.

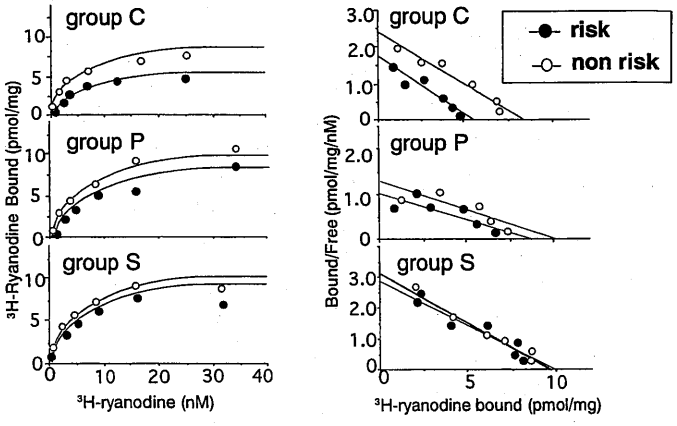

Fig. 3 Graphs showing representative plots of high-affinity ^{3}H-ryanodine binding determined at pCa 4.7 in the microsomal fraction enriched by SR obtained from area at risk (●) and non risk (○). On the left panels, saturation binding curves are shown; on the right panels, Scatchard plots are shown. Group C: control undergoing a single 20-minute ischemia; Group P: preconditioned group followed by 20-minute ischemia; Group S: preconditioned group without 20-minute ischemia.

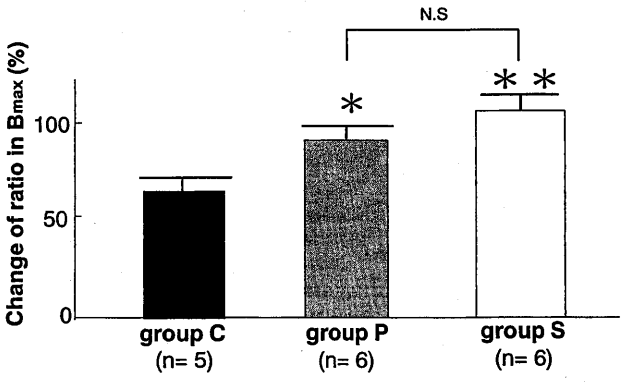

Fig. 4 Ratios in Bmax of areas at risk against those of areas at non risk are shown. A significant improvement in the ratio of Bmax in group P is indicated when compared to group C. The preconditioning procedure itself (group S) did not produce a reduction in the ratio of Bmax. *;P<0.05, **;P<0.01 vs. group C.

risk and non risk among the groups. Fig 4 showed the ratio of Bmax in area at risk to area at non risk. Compared to control group, this ratio was significantly higher in the preconditioned group P. Fig. 5 shows Ca^{2+} dependence of ryanodine binding. At each Ca^{2+} concentration, ^{3}H-ryanodine binding of risk area in group C was lower than those of risk or non-risk area in group P.

Discussion

Zucchi et al.[12] showed that a brief ischemia followed by reperfusion as ischemic preconditioning (IP) caused reduction of the density of ryanodine receptors (Bmax). They indicated this downregulation of ryanodine receptors brought by IP could be one of the mechanisms of cardioprotection through attenuating the

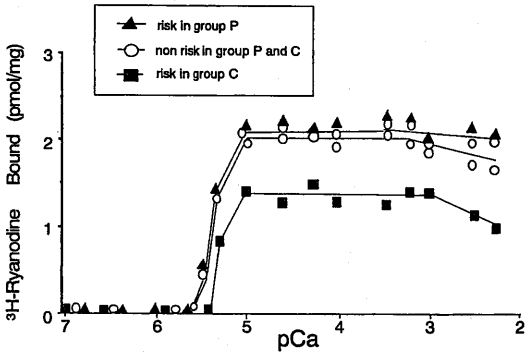

Fig. 5 Graphs showing Ca^{2+} dependence of high-affinity ^{3}H-Ryanodine binding to microsomal fraction enriched in SR. At pCa=4.8. the ryanodine binding reached plateau in each group. The ryanodine binding in risk area of control was consistently lower than that of other groups.

Ca^{2+} overload. The purpose of the present study was to test 1) whether a brief ischemia / reperfusion causes a reduction of ryanodine receptors in dog hearts and 2) to evaluate the effect of IP on ryanodine receptor density after 20-minute sustained ischemia. It has been reported that myocardial necrosis did not occur within 20 minutes ischemia in dogs[5]. Thus we could avoid the possibility that the alteration in Bmax during sustained ischemia was caused by the artifact induced by the necrotic tissue when the purified fraction with myofibrillar protein was utilized[30]. Ryanodine has been shown to specifically bind to sarcoplasmic reticulum (SR) foot process Ca^{2+} releasing channels[31,32]. In myocardial tissue, ryanodine binds to the Ca^{2+} channels with both high and low affinity[33]. Nanomolar concentrations of ryanodine bound tightly to the high-affinity receptors[33] keeps the Ca^{2+} releasing channels in open state[29,34]. Ryanodine binding in micromolar concentrations to a low-affinity site is responsible for closure of the calcium releasing channels[34,35]. In this study, we utilized the high-affinity binding of ryanodine to detect the number of the calcium releasing channel.

Without preconditioning, 20 minutes no flow ischemia reduced Bmax of the ischemic myocardium compared with that of non-ischemic myocardium, in good accordance with previous reports in dogs[24]. However, ischemic preconditioning significantly attenuated the reduction in Bmax in the ischemic myocardium, indicating the protective effect of ischemic preconditioning to ryanodine receptors. In contrast with the study by Zucchi et al.[12] who reported the reduction in Bmax occurred immediately after IP, our study showed no down regulation of ryanodine binding sites after brief ischemia/reperfusion. The reason of this controverse between our data and that of Zucchi et al could be attributed to the following 3 reasons; 1) the difference of the regimen of ischemic preconditioning, 2) difference of the experimental conditions, i.e., in situ versus Langendorff-perfused heart, 3) species difference. We obtained a substantial effect of ischemic preconditioning in view of the attenuation of the ryanodine receptor. So the difference of the ischemic preconditioning procedure should not be a major reason for the discrepancy. Experimental condition could be important since the Langendorff-perfused heart did not receive blood which includes catecholamines and cytokines and many other factors which influence the myocyte metabolism. However, the most influential factor could be the species difference. It is suggested that there are differences in signaling pathways which trigger preconditioning between animals. For example, adenosine and ATP-sensitive potassium channels play key roles in rabbits[7,36] and dogs[11,37] while they were less important in rats[9]. These observations suggested that there may be fundamental differences in the mechanism of IP between dogs and rats. Taking these previous studies and our results into consideration, it could be implicated that as the possible mechanism of IP, adenosine and ATP-sensitive potassium channels play key roles in dogs, but not in rats, whereas, ryanodine receptor down regulation plays key roles in rats, but not in dogs. Although our study indicated that IP is not mediated by down regulation of Ca^{2+} release channels during brief ischemia/reperfusion in dogs, there is evidence that the pathophysiology of IP is related to the control of Ca^{2+} homeostasis in dog model[38]. During ischemia, cytosolic free Ca^{2+} increases by several mechanisms[13-17]. 1)

reduction of ATP phosphorylation potential which inhibits Ca^{2+} transport by the sarcoplasmic reticulum $Ca^{2+}-ATPase$; 2) Ca^{2+} influx caused by the $Na^{+}-Ca^{2+}$ exchanger as a consequence of increased intracellular Na^{+} induced by $Na^{+}-H^{+}$ exchange, 3) Ca^{2+} released from the SR in calcium dependent manner. Our results suggest that the attenuation of calcium overload caused by IP should be mediated by some other mechanisms than SR Ca^{2+} release channel down regulation in dogs.

Stunned myocardium is defined as contractile dysfunction of viable myocardium reperfused after a period of ischemia[4]. A decrease in the Ca^{2+} sensitivity of contractile proteins could be an underlying mechanism of stunned myocardium[40]. Alternatively deficiency in the handling of cytosolic Ca^{2+} [21,22] would be an another involved mechanism of stunned myocardium. Previous studies done in dogs showed that IP improved myocardial contractile function after reperfusion as well as reducing infarct size[39]. In the present study, in preconditioned group, ryanodine binding sites were significantly preserved after 20- minute ischemia compared with non- preconditioned group. Improved $Ca^{2+}-ATPase$ activity was previously reported during subsequent ischemia when preconditioned[39]. Thus the available Ca^{2+} released and re- uptaken from the SR should be increased when preconditioned and this should contribute to the improved contractile function after reperfusion by preconditioning.

We should point out some limitations in the present study. First, in our experiments, specimen obtained from risk and non risk area were purified and incubated with ^{3}H-ryanodine under identical conditions; the pH and concentrations of Ca^{2+}, K^{+}, and Mg^{2+} modifying ^{3}H-ryanodine binding were identical. Therefore the binding assay performed in the present study did not necessarily reflect the ryanodine binding density in situ, since free Ca^{2+} concentration and the other ion concentration should vary during ischemia. Secondly, varying degree of collateral flow exists in dogs and this may cause the variability of the data when ischemia was created by coronary occlusion[41,42]. We used beagle dogs, which have less collateral vessels than mongrel dogs, and ligated visible collateral vessels to the area at risk. With these procedures, coronary occlusion caused bulging of risk area and we ascertained that the myocardial blood flow determined with colored microspheres was less than 0.1 ml/min/g in the subendocardial region at risk.

In conclusion, we assessed the effects of 5 minutes ischemia/reperfusion on the ryanodine receptors before and after 20 minutes subsequent ischemia in dogs. The brief ischemia and reperfusion as ischemic preconditioning did not down-regulate the ryanodine binding density. However, when preconditioned the decrease in ryanodine binding density of risk area was significantly attenuated after subsequent 20 minutes ischemia. Further study should be needed to show the time course of the recovery of ryanodine binding density after reperfusion together with the recovery of the contractile function with and without IP. Moreover, it is important to clarify the molecular mechanism responsible for ryanodine binding site alteration caused by ischemia with and without IP.

Acknowledgment

I thank Dr. Toshiro Miura, Dr. Tomoko Ohkusa and Prof. Masunori Matsuzaki for helpful advice and discussion all over my study. And I also thank Dr. Shuji Kawamura for his skillful technical cooperation of this study.

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