Original

Alterations in Sarcoplasmic Reticulum and Mitochondrial Functions in Stunned Myocardium: Relation between Regional Myocardial Function and Biochemical Analyses

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Abstract: We studied the contribution of intracellular microorgan dysfunction in the post ischemic stunned myocardium. In 47 mongrel dogs, LAD occlusion for 15 min (I-15m) and subsequent reperfusion for 60 min (R-60m) were performed with simultaneous records of hemodynamics, coronary blood flow (CBF), regional myocardial blood flow (MBF) and segment shortening (SS). Sarcoplasmic reticulum (SR) and mitochondria (Mt) were analyzed using biochemical procedures. Hemodynamics indicated no remarkable changes throughout the experiment. CBF showed reactive hyperemia to 370% of control transiently after reprefusion, then returned to control level at R-15m. SS diminished to -22% of control at I-15m, and it was still depressed at 58% at R-60m (stunning). Ca-ATPase activity of SR was reduced to 57% of control at I-15m, and remained depressed at 78% at R-60m. State III respiration of Mt decreased to 75% of control, and dinitrophenol-stimulated ATPase activity of Mt was 72% of control at I-15m. These activities did not recover completely at R-60m (77% and 75%, respectively). Both Ca-ATPase activity of SR and state III respiration of Mt were positively correlated to SS. SDS-gel electrophoresis of SR protein revealed no irreversible changes. These results suggested that contractile dysfunction after brief ischemia is related to the energy demand and supply, and the transient dysfunction of microorgans is one of the main causes of stunned myocardium.

Key words: stunned myocardium, reperfusion injury, sarcoplasmic reticulum, mitochondria, canine heart

Introduction

Reperfusion in the early stage of myocardial infarction is expected to improve the prognosis of patients of acute myocardial infarction (aMI). Percutaneous transluminal coronary angioplasty (PTCA) and percutaneous transluminal coronary recanalization (PTCR) have already been established in clinical practice. Reperfusion in the early stage of myocardial infarction has several complications, however, such as reperfusion arrhythmia, vascular damage, no-reflow phenomenon and contractile dysfunction. Reperfusion injury after a brief period of myocardial ischemia termed "myocardial stunning"¹⁾, has prolonged, reversible regional contractile dysfunction without cell necrosis. Several hypotheses have been proposed as mechanisms of stunning. These include depletion of high energy phosphate^{2,3)}, dysfunction of sarcoplasmic reticulum⁴⁻⁶⁾, depressed calcium activation of contraction⁷⁾, calcium over-

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load⁸⁾, and generation of free radicals⁹⁾. However all of these hypotheses remain inadequately explained. For example, a depressed exchange rate of cytosolic ADP for intramitochondrial ATP facilitated by adenine nucleotide translocase may be associated with a depressed rate of ATP synthesis by the mitochondria. The loss of mitochondrial respiration cannot be explained by the loss of adenine nucleotide translocase activity, because uncoupling between electron transport and ADP phosphorylation did not stimulate respiration above state III levels and oxidant stress can increase the permeability of mitochondria to calcium and lead to uncoupling^{10,11)}. Other reports have stated that the sarcoplasmic reticulum shows a decrease in its Ca^{2+} accumulating activity with a reduction in the activity of the Ca^{2+} , Mg^{2+} -ATPase. A decrease in the amount of calcium stored in the sarcoplasmic reticulum as a result of a reduction in the calcium pump activity could diminish contractile protein activation through attenuated calcium release during systole¹²⁾, while the activities of the sarcolemmal Na^+ , K^+ -ATPase and Ca^{2+} -ATPase enzymes, and of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and Na^+/H^+ antiporter remain fully functional. In the present study, we investigated the relation of the contractile functions in the stunned myocardium and the damage to microorgans, such as sarcoplasmic reticulum (SR) and mitochondria (Mt).

Materials and Methods

1) Instrument; Forty-seven mongrel dogs weighing 8 to 22 kg were anesthetized with intravenous injection of urethane (450 mg/kg), α -chloralose (45 mg/kg) and diazepam (10 mg). A left thoracotomy was performed under positive pressure respiration with room air using a Harvard type ventilator. The left anterior descending coronary artery (LAD) was isolated and a Doppler flow velocity probe (Triton Technology Inc., San Diego) was placed between the first and second diagonal branch to measure the coronary blood flow (CBF). Pig tail and Swan-Gantz catheters were inserted into the femoral artery and saphenous vein, and heart rate (HR), left ventricular systolic pressure (LVP), LV dp/dt, pulmonary arterial pressure (PA) and cardiac output (CO) were recorded by the polygraph system (Nihon Kohden C.O., Tokyo). Regional myocardial blood flow (BMF) was determined by the H_2 gas clearance method using a platinum electrode (Unique Medical Co. Ltd., Tokyo) inserted into subendocardial muscles of the ischemic (I) and the non-ischemic (NI) myocardium. Myocardial regional function was measured by a pair of piezoelectric crystals (Triton Technology Inc., San Diego) inserted at a depth of 7–9 mm into the subendocardium. Depths of crystal were verified at the end of each experiment. The distance between the two crystals was measured, and percent segment shortening (%SS) was calculated using the equation,

$$\%SS = (\text{EDSL} - \text{ESSL}) / \text{EDSL} \times 100, \text{ where}$$

ESSL is end systolic segment length, and EDSL is end diastolic segment length.

2) Experimental protocol; LAD was occluded by the snare for 15 min and subsequent reperfusion was performed for 60 min with continuous records of hemodynamics (HR, PAP, CO, LVP, LVEDP, and LVdp/dt), CBF and MBF.

3) Biochemical analyses; All of the procedures were carried out at 4°C unless otherwise mentioned, and all the reagents were of reagent grade. The beating heart was removed at the end of 15 min ischemia, and after 15 to 60 min reperfusion, washed briefly with ice cold saline solution, and placed on crushed ice. The ischemia-reperfused and non-ischemic (control) myocardium were reserved and divided into subendocardium (Endo) and subepicardium (Epi).

a) Preparation of the SR fraction; SR was extracted as the light microsomal fraction using the modified method of Harigaya-Schwartz¹³⁾. Briefly, three grams of heart muscle were minced with scissors and homogenized with a polytron homogenizer (Kinematica PT10/35) with 9 volumes of a solution containing 0.01 M NaHCO_3 , 0.05 M NaN_3 , and 0.001 M ethylene glycol tetraacetic acid (EGTA). The homogenate was centrifuged at $9,000 \times g$ (Hitachi himac CR21) for 10 min and the supernatant was centrifuged again at $12,000 \times g$ for 10 min to remove the lysosome rich fraction. The resulting supernatant was ultracentrifuged at $120,000 \times g$ (Hitachi 55P-72) for 45 min twice. The precipitate obtained was regarded as SR. The SR fraction was suspended in 0.05 M KCl and 0.02 M N-trismethyl 2-aminoethane sulfonic acid (TES)-tris (pH 7.0), and the protein concentration was determined by the biuret procedure.

b) Ca^{2+} -stimulated ATPase activity and SDS gel electrophoresis of SR; Ca^{2+} -stimulated ATPase activity of SR was determined according to the modified method of Harigaya and Schwartz¹³⁾, which was expressed as a difference of the activities in the presence of 2×10^{-5} M CaCl_2 or 0.001 M EGTA in the solution containing 0.1 M KCl, 0.01 M MgCl_2 , 0.005 M NaN_3 , 30 mM ATP and 0.02 M TES-tris (pH 7.0) at 37°C for 15 min. Inorganic phosphate liberated from ATP was measured by the modified method of Fiske-SubbaRow¹⁴⁾.

SDS Gel electrophoresis of SR composing proteins; electrophoresis of SR proteins was carried out in the presence of SDS with 10% polyacrylamide by the modified method of Laemmli using silver stain¹⁵⁾.

c) Preparation of mitochondria; Mitochondria were prepared by the modified method of Sordahl and Stewart¹⁶⁾ utilizing alkaline protease (Nagarse, Nagase Biochemicals Ltd., Tokyo) as follows. Briefly, three grams of heart muscles were minced with scissors and homogenized with 9 volumes of a solution containing 0.25 M sucrose, 0.01 M EGTA, and 0.03 M tris-HCl (pH 7.2) with a polytron homogenizer and centrifuged at $27,000 \times g$ (Hitachi himac CR21) for 10 min. One mg of Nagarse per gram of starting heart tissue was added to the pellet and incubated at 4°C for 8 min, and 40 ml of a solution containing 0.18 M KCl, 0.01 M EGTA, 0.5% (wt/vol) bovine serum albumin (BSA), and 0.03 M tris-HCl (pH 7.2) were added, then homogenized twice with a motor driven teflon pestle and centrifuged at $500 \times g$ for 5 min. After filtration using cheese cloth, and centrifugation twice at $12,000 \times g$ at 10 min a solution containing 0.18 M KCl, and 0.03 M Tris-HCl (pH 7.2), 0.5% (wt/vol) was added for a final concentration of approximately 15 mg/ml.

d) Oxidative phosphorylation of mitochondria; Mitochondrial oxidative phosphorylation was measured with an oxygen electrode (Oxygen Consumption Recorder PO-100A, Yanagimoto Mfg. Co. Ltd., Kyoto), according to the method of Hagihara¹⁷⁾. The reaction medium contained 0.01 M KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM MgCl_2 , 0.25 M sucrose and 0.02 M potassium phosphate buffer (pH 7.2) at 25°C . Succinate or glutamate plus malate were used as substrates and ADP was added to estimate oxidative phosphorylation. Oxygen consumption rates in states III and IV were determined from a polarographic record by the method of Chance and Williams¹⁸⁾.

e) ATPase activity of mitochondria; Dinitrophenol (DNP)-stimulated ATPase activity of mitochondria was examined by the modified method of Pullman et al.¹⁹⁾ in a reaction medium containing 0.21 M sucrose, 0.02 M KCl, 0.02 M tris-malate (pH 7.2) and 5 mg of mitochondria. The reaction was started by the addition of 0.001 M DNP and 0.03 M ATP at 25°C , and the amount of phosphate liberated in the reaction was assayed by the method of Fiske and SubbaRow¹⁴⁾.

4) Statistical Analyses; Results were expressed as mean $\pm$ standard deviation of experimental indicators. ANOVA for repeated measurements was used to determine the significance of changes during time-dependent multiple observations. Student's *t*-test was used to compare changes between the two groups, where *p* values less than 0.05 were considered as significantly different.

Results

Hemodynamics; During 15 min occlusion and subsequent reperfusion for 60 min, no significant hemodynamics (HR, PAP, CO, LVP, LVEDP, and LVdp/dt) changes occurred (Fig. 1).

Coronary blood flow (CBF) and regional myocardial blood flow (MBF); CBF and MBF of ischemia-reperfused area were summarized in Fig. 2. No significant changes of CBF and MBF were observed in the non-ischemic region throughout the experiment. CBF was 25.0 ± 12.6 ml/min in the preocclusion (control). During coronary occlusion, CBF indicated zero, and at just after reperfusion CBF showed transient reactive hyperemia of about 370% (92.9 ± 51.9 ml/min) ($P < 0.01$) of the control value, and then returned to control level after 15 min reperfusion. MBF was 114.0 ± 19.1 ml/100 g/min in the control. During ischemia, MBF decreased remarkably to $19.0 \pm 9.9\%$ ($P < 0.01$) of control. MBF showed transient reactive hyperemia to $134.2 \pm 17.5\%$ ($P < 0.01$) at 5 min reperfusion. After that, it was reduced to below the control value, and indicated $75.4 \pm 18.5\%$ of control at 60 min reperfusion.

% Segment shortening (%SS); Regional myocardial contractile functions indicated by % segment shortening (%SS) are summarized in Fig. 3. No significant changes occurred in the

Fig. 1. Hemodynamic changes during ischemia and reperfusion.

HR; heart rate, PAP; pulmonary artery pressure, CO; cardiac output, LVP; left ventricular pressure, LVEDP; left ventricular end-diastolic pressure, Max LVdp/dt; max left ventricular dp/dt.

Fig. 2.

Fig. 3.

- Fig. 2. Coronary blood flow and myocardial blood flow.
 (A); coronary blood flow of left anterior descending artery during ischemia and reperfusion.
 (B); myocardial blood flow of ischemia-reperfusion regional myocardium.
 * $p < 0.05$, ** $p < 0.01$ vs non-ischemia.

- Fig. 3. % Segment shortening during ischemia and reperfusion.
 ●—●; Ischemic myocardium. ■—■; Non-ischemic myocardium.
 Plots indicate % of control.

NI region throughout the experiment. In the ischemic myocardium at the end of 15 min ischemia, %SS showed $-22.5 \pm 30.5\%$ ($P < 0.01$) of previous control, indicating dyskinesia and bulging of the myocardium. At 5 min reflow, %SS showed transient improvement to $59.2 \pm 15.5\%$, however, %SS decreased again to $40.2 \pm 26.3\%$ at 15 min reflow, and $54.2 \pm 30.2\%$ ($P < 0.01$) at 60 min reflow, which implied stunning.

Ca^{2+} -ATPase activity of SR; Ca^{2+} -ATPase activities of the SR fraction are shown in Fig. 4. Yields of SR protein did not differ between the ischemia-reperfusion and non-ischemic myocardium. In the non-ischemic myocardium, Ca^{2+} -ATPase activity was $10.9 \pm 1.4 \mu\text{moles Pi/mg protein/hr}$ in Endo, and 13.3 ± 2.1 in Epi. Ca^{2+} -ATPase activity at 15 min ischemia was significantly reduced to 5.7 ± 1.3 ($P < 0.01$) in Endo and 6.0 ± 1.4 ($P < 0.01$) in Epi. After the reflow, this activity in Endo still depressed to 8.0 ± 0.7 ($P < 0.05$) at 60 min reperfusion. The activity in Epi was also still depressed at 9.2 ± 1.7 ($P < 0.05$) at 60 min reperfusion. The relation between Ca^{2+} -ATPase activity of SR and %SS showed a significant and positive correlation (Fig. 5), and this suggested that the Ca uptake of SR was related to the regional myocardial function after the short period of ischemia.

SDS gel electrophoresis of SR; Electrophoresis of SR is shown in Fig. 6. A major ATPase protein was identified with the standard of about 100,000 daltons. There were neither compositional changes in the major ATPase protein nor the appearance of the new band in all cases.

Fig. 4. Ca⁺⁺-ATPase activity of SR.

□; subendocardium. ■; subepicardium. * $p < 0.05$, ** $p < 0.01$ vs non-ischemia.

Fig. 5. Correlation between Ca⁺⁺-ATPase activity of SR and % Segment shortening (SS).

(A); Ca⁺⁺-ATPase activity of subendocardium vs %SS.

(B); Ca⁺⁺-ATPase activity of subepicardium vs %SS.

Fig. 6. SDS gel (10% acrylamide) electrophoresis of SR protein. Molecular weight standards were as follows; 116 kDa: β -galactosidase. 53 kDa: glutamic dehydrogenase. Major band (=100 kDa) indicates ATPase protein.
A; Non-ischemia, B; 15 min ischemia, C; 15 min ischemia and 60 min reperfusion, D; Molecular Marker.

Mitochondrial respiratory activity; The oxygen consumption rates in state III of mitochondria, using succinate or glutamate plus malate as substrates, are presented in Fig. 7. In the non-ischemic myocardium, state III respiratory activities were $197.5 \pm 27.4 \mu\text{m atoms O/mg protein/min}$ in Endo and 199.5 ± 33.6 in Epi, using succinate as the substrate. State III respiratory activities with glutamate plus malate as the substrate indicated 198.4 ± 42.7 in Endo and 207.6 ± 39.3 in Epi, respectively. At 15 min ischemia, state III respiratory activity of mitochondria using succinate was reduced significantly to $75.4 \pm 17.7\%$ ($P < 0.05$) in Endo, and $71.3 \pm 13.9\%$ ($P < 0.05$) in Epi compared with non-ischemic myocardium. These activities showed $79.1 \pm 8.6\%$ ($P < 0.05$) and $73.5 \pm 9.1\%$ ($P < 0.05$) of control at 60 min reflow, respectively. The respiratory consumption index (RCI) revealed changes similar to the state III respiratory activity and the oxygen consumption rate in state IV indicated no significant change during ischemia-reperfusion (data not shown). Using glutamate plus malate as the substrate, state III respiratory activities indicated patterns similar to those with succinate as the substrate, ($72.6 \pm 16.7\%$ ($P < 0.05$) in Endo and $73.3 \pm 13.4\%$ in Epi at 15 min ischemia), showing $87.1 \pm 28.0\%$ in Endo and $76.4 \pm 26.4\%$ in Epi at 60 min reflow, respectively. The relation between mitochondrial state III respiratory activity in succinate and % segment shortening showed a significant positive correlation (Fig. 8). This suggested that the mitochondrial respiratory function was also related to regional myocardial function of the stunned myocardium.

Dinitrophenol (DNP)-stimulated ATPase activity; DNP-stimulated ATPase activities are shown in Fig. 9. In the non-ischemic myocardium, this activity indicated 11.2 ± 1.27 in

Fig. 7. State III respiration of mitochondria.

(A); succinate was used as the substrate.

(B); glutamate plus malate was used as the substrate.

□; subendocardium. ■; subepicardium. * $p < 0.05$, vs non-ischemia.

Epi. In the 15 min ischemia, this activity was reduced significantly to $76.1 \pm 11.2\%$ ($P < 0.01$) of control in Endo. The low activity continued at 60 min reflow, of which the value was $79.0 \pm 8.0\%$ ($P < 0.05$) of control. DNP-stimulated ATPase activity in Epi decreased to $73.6 \pm 13.5\%$ ($P < 0.01$) of control 15 min. This activity did not recover at 60 min reflow ($74.4 \pm 6.4\%$ ($P < 0.05$)).

Discussion

Following a brief period of ischemia, prolonged ventricular dysfunction without necrosis, is a phenomenon known as "stunned myocardium"²¹. In stunned myocardium, the situation is defined as reduced myocardial function, normal coronary blood flow, full recovery outcome after a period of hours to several days, and reversible ischemic injury. The mechanisms responsible for myocardial stunning have been pursued, but the single cause has not been established. A number of mechanisms have been suggested, such as reduced ability to resynthesize high-energy phosphates²⁰), impaired sympathetic neural responsiveness²¹), heterogeneous impairment of regional perfusion, loss of creatine kinase activity and impaired utilization of energy by the myofibrils²²), leukocyte activation²³), decreased sensitivity of the myofilaments to calcium²⁴), transient intracellular calcium overload⁸), and free radicals⁹). However all hypotheses remain unclarified.

The experiments in this study showed that during 15 min ischemia, %SS reduced from -20% to -30% of the previous control, however, after transient improvement, prolonged postischemic ventricular dysfunction from 50% to 60% continued at 60 min reflow (stun-

Fig. 8. Correlation between state III respiration of mitochondria (succinate as the substrate) and % Segment shortening (SS).
 (A); State III respiration of subendocardium vs %SS.
 (B); State III respiration of subepicardium vs %SS.

Fig. 9. Dinitrophenol (DNP)-stimulated ATPase activity of mitochondria.
 □; subendocardium. ■; subepicardium. ** $p < 0.01$, * $P < 0.05$ vs non-ischemia.

ning). This reversible myocardium had no irreversible ultrastructural observation recognizable such as mild intracellular edema and slight mitochondrial swelling that have been reported likely to be the reversible changes; there was no appearance of mitochondrial dense deposits and contraction band necrosis which have been regarded to be irreversible changes (not shown). On the other hand, in our previous studies, we reported that the reduction of Ca^{2+} -ATPase activity of sarcoplasmic reticulum (SR), as an energy demand system, with

degradation of major ATPase protein and phospholipids of SR and a decrease of mitochondrial respiratory activities, as an energy supply system, are the indexes of ischemic irreversible changes in experimental acute myocardial ischemia, as early as at 20 min of coronary occlusion in Endo²⁵⁾. These findings supported that the duration of 15 min ischemia is a reasonable period for the experimental model of myocardial stunning.

The data of the present paper suggest that the decrease of Ca^{2+} -ATPase activity of SR, which is the major energy consuming enzyme next to myosin ATPase, decreased of Ca -uptake⁶⁾ and the inhibitor of Ca -release and Ca -coupling to myocyte with the reduction of Ca storage of SR⁵⁾. Then a decreased sensitivity of the myofilaments to cytosolic free Ca support the excitation contraction uncoupling⁷⁾ due to dysfunction of SR. Also these results suggested that the decrease in the amount of calcium stored in the SR as a result of a reduction in the calcium pump activity and inhibition of the uptake of calcium by SR could diminish contractile protein activation and inhibition of uptake of calcium through attenuated calcium release during systole. We speculated that the mechanisms of inactivation of Ca^{2+} -ATPase without protein degradation were the decreased Ca^{2+} sensitivity of the myofibrils or an uncoupling between the Ca^{2+} -ATPase and the cross-bridge cycles.

On the other hand, the mitochondria can play an important part in energy production and in the regulation of intracellular calcium. During ischemia the absence of oxidative phosphorylation results in net degradation of ATP to ADP and AMP. Cytosolic AMP degraded to nucleosides and purine bases results in the net loss of adenine nucleotide in the cytosol. Adenosine may replenish the mitochondrial ATP pool, which is essential for ATP synthesis, although only comprising 15–20% of the total cellular pool¹¹⁾. Mitochondria maintain the viability of reoxygenated cells by buffering changes in cytosolic Ca^{2+} . The oxygen dependent influx of cytosolic Ca^{2+} is probably mediated through the $\text{Na}^+/\text{Ca}^{2+}$ exchange protein. Metabolic recovery occurs with partial restoration of ATP levels²⁶⁾, and reoxygenation is associated with a decrease in the efficiency of electron transfer at the level of NADH-CoQ reductase in the mitochondrial inner membrane and an increase in the intramitochondrial Ca^{2+} content²⁷⁾. The present study showed that at after 15 min ischemia, mitochondrial respiration was decreased significantly, and this reduced respiration continued after 60 min reperfusion. These data support that the stunning was related to the reduced function of mitochondria, which are the major energy production. During ischemia, intracellular levels of ATP are decreased, and the decreased level of cellular ATP persisted for a long time in stunned myocardium. Between the change of the ATP level and contractile recovery, similar time course are permitted²⁸⁾. The delayed resynthesis of ATP is in turn the probable consequence of the loss of adenosine⁵⁾ and related compounds during the ischemic period. These results indicated that the attribution of mitochondrial respiratory dysfunction and uncoupling between electron transport and mitochondrial ATPase played a role in ATP depletion²⁹⁾.

In subsequent experiments, these reduced activities of SR and Mt and regional myocardial function recovered to the control values at 7 days reperfusion³⁰⁾. So we suggested that these biochemical analyses of subcellular microorgans were suitable to clarify the mechanism of myocardial stunning.

We conclude that the contractile function after brief reversible ischemia is correlated to SR and Mt functions that indicate indexes of energy demand and supply, and SR and Mt dysfunctions are one of the main causes in stunned myocardium.

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