DEGRADATION OF THE CARDIAC SARCOPLASMIC RETICULUM IN ACUTE MYOCARDIAL ISCHEMIA

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The degradation of the sarcoplasmic reticulum (SR) in acute myocardial ischemia was studied with references to the regional irreversibility and to the mechanism of ischemic degradation by the measurements of Ca"-stimulated ATPase activity and composition of the major ATPase protein of the SR and activity of cathepsin B of the SR and lysosome (Ly) fractions.

Ca"-stimulated ATPase activity decreased to 66% of that of the non-ischemic portion at 20 min after coronary ligation in the subendocardium (Endo) and to 44% at 30 min in the subepicardium (Epi). Composition of the major ATPase protein decreased to 55% and 73% at 30 min in Endo and Epi, respectively. In both SR and Ly fractions cathepsin B exhibited the maximal activity at 6.0–6.5, and pH dependent. And incubation of the SR at pH 6.0 induced the degradation of the ATPase protein quite similarly to that in vivo ischemia.

These results suggest that the degradation of the SR membrane of ischemic myocardial cells begins earlier in Endo 20 to 30 min after the cease of the coronary blood flow, and extends to Epi later. Cathepsin B is strongly conceivable to play an initial role of necrotic process of the ischemic myocardial cells by activation inside of the SR in ischemic acidic state.

Because the ischemic myocardium changes irreversibly after a certain period following the cessation of coronary blood flow, it is very important to identify this point of no return in order to adopt measures, such as coronary revasculization, that will salvage the ischemic heart muscle.

Evidence of fine structural irreversible changes was observed by Jennings and co-workers at between 40 to 60 min after coronary occlusion. However, in clinical practice, many cardiologists report the appearance of myocardial necrosis in ischemia for 15 to 20 min after 24 hours. Similarly, angina pectoris of longer than 20 to 30 min is usually expected to result in the occurrence of myocardial infarction. It is quite possible that the irreversible changes appear in the microorgans of the ischemic myocytes prior to the morphologic evidence of ischemic necrosis.

Recently the activation of acid hydrolases in the ischemic myocardium was identified as one of the causes of ischemic myocardial injury; wildenthal and the associates have called this phenomenon "the lysosomal hypothesis". Originally the localization of these acid hydrolases was thought to be inside the lysosomes (Ly). However, Hoffstein and co-workers noted that these acid hydrolases were also located in the sarcoplasmic reticulum (SR) and, in addition,
that the action of the acid hydrolases in the SR was more intensely related to the necrosis of the ischemic myocytes.

We have reported in previous articles that the degradation of the major ATPase protein of the SR occurred in the early ischemic myocardium, and we regarded this phenomenon as one of the earlier signs of myocardial irreversibility. We also verified the activation of these acid hydrolases inside of the SR both biochemically and cytochemically.

Based on these facts, we studied the regional changes in the SR in the earlier ischemic myocardium in relation to the mechanism of the degradation focused on cathepsin B which is one of the well-known lysosomal acid hydrolases and regarded as the initial inducer of the ischemic degradation.

MATERIALS AND METHODS

Animal experiment; Thirty mongrel dogs weighing 8 to 14 kg were anesthetized with intravenous injection of sodium pentobarbital (Nembutal®) at 30 mg/kg body weight. A left thoracotomy was performed under a positive pressure respiration using a Harvard type respirator, and the anterior descending branch of the left coronary artery (LAD) was excised and totally occluded with silk thread just below the first diagonal branch. Electrocardiogram was recorded and the occurrence of severe transmural ischemia was confirmed by the continuous ST segment elevation of greater than 0.5 mV in the leads near V3 and V4. Ten to 120 min after LAD ligation, the beating heart was removed rapidly and washed briefly with ice-cold saline solution and placed on crushed ice. The left ventricular free wall of the LAD area was reserved as a sample, together with the left ventricular posterior wall as a non-ischemic control. Heart muscle was divided at the middle of the wall to the subendocardial (Endo) and subepicardial (Epi) layers.

Preparation of the SR and Ly fractions; All of the procedures were carried out at 4°C unless otherwise mentioned. A 2.5 g portion of heart muscle was minced with scissors and homogenized with Polytron homogenizer with 9 volumes of a solution containing 0.01M Na2HPO4 and 0.0001M EGTA. The homogenate was centrifuged at 8,000 x g for 10 min and the supernatant was centrifuged again at 12,000 x g for 10 min. This precipitate of the lysosome-rich fraction was suspended in 0.05M KCl and 0.02M N-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES)-tris (pH 7.0) and was spun again at 12,000 x g for 10 min to eliminate the contamination of the soluble fraction. The supernatant of the first centrifugation at 12,000 x g was centrifuged at 120,000 x g for 30 min twice. The resulting precipitate of the microsome fraction was regarded as the SR. Each pellet of the Ly and SR fractions was suspended in 0.05M KCl and 0.02M TES-tris (pH 7.0). Protein concentration was determined by the method of Lowry.

Ca**+ stimulated ATPase activity; Ca**+ stimulated ATPase activity of the SR was determined according to the modified method of Harigaya et al. Ca**+-ATPase activity was expressed as the difference of the activities in the presence of 2 x 10^-5M Ca**+ and 0.001M EGTA in 0.05M KCl, 0.001M MgCl2, 0.005M Na2EDTA, 0.05M ATP and 0.02M TES-tris (pH 7.0) at 37°C for 10 min. The liberated phosphate was determined by the modified method of Fiske and Subbarow.

Activity of cathepsin B; Activity of cathepsin B was determined in the SR and Ly fractions according to the modified method of Strewler et al. The reaction mixture containing potassium phosphate (pH 6.5), 0.005M EDTA, 0.005M cysteine in a total volume of 1.0 ml. Reaction was started with the addition of 0.003M α-N-benzoyl-DL-arginine-2-naphthylamide as a substrate at 40°C for 15 min and stopped by the addition of 0.2M glycine-NaOH (pH 10.5). The activity of cathepsin B was expressed as liberation of β-naphthylamine by fluorescence colorimetry (Ex 365–Em 405 nm). Effect of pH on cathepsin B activity was studied at a series of pH from 5.0 to 8.0 in potassium phosphate buffer.

Gel electrophoresis of the SR proteins; Electrophoresis of the SR proteins was carried out in the presence of SDS by the modified method of Weber and Osborn. Composition of the SR proteins was determined from a densitometric curve of a gel stained with Coomassie Brilliant Blue.

In vitro study of the SR in the acidic state; The SR from the non-ischemic myocardium was suspended in solutions with a series of pH from 4.0 to 8.0 containing 0.05M KCl and 0.005M EGTA, and incubated at 37°C for 60 min. Then compositions of the SR proteins were determined by SDS gel electrophoresis.

Statistics; Results were expressed as mean ± 1SD. Statistical analysis of the data was done
Fig.1. Electron micrographs of the lysosome rich (A) and the SR fractions (B). Bars indicate 0.2 μ.

Fig.2. Yields of proteins of the SR and Ly fractions contained in 1g wet weight myocardial tissue. 
A: endocardial muscle (Endo); B: epicardial muscle (Epi); Bars indicate mean ± SD. The bars with slant lines and the closed bars indicate the SR and Ly fractions, respectively.

according to t-test, and a p value of less than 0.05 was regarded to be significantly different.

RESULTS

Electron microscopic observation of the SR and Ly fractions; Fig.1 shows electron micrographs of the lysosome rich and the microsome fractions. The lysosome rich fraction was constituted of many large-sized vesicles and the figure was similar to that of in the previous paper. The microsome fraction contained many fragments of vesicles of the SR. In both fractions, contamination of mitochondria was very small.

Yields of proteins; Yields of proteins of the SR and Ly fractions are shown in Fig. 2. In the non-ischemic Endo, the yields of the SR fraction was 0.56 ± 0.06 mg/g wet weight and that of the
Fig. 3. Ca\(^{2+}\)-ATPase activity of the SR from Endo and Epi in acute myocardial ischemia expressed as total activity/g wet weight/hr. Bars indicate mean ± SD. The open circles and the closed circles indicate the activities in Endo and Epi, respectively. *p < 0.05, **p < 0.01 against control value.

Fig. 4. Electrophoretic gels of the SR from Endo and Epi (10% acrylamide).

Ly fraction was 0.30 ± 0.05, and in the Epi they were 0.58 ± 0.08 and 0.33 ± 0.07, respectively. In the ischemic myocardium, the yield of proteins in each fraction was no different from that of the non-ischemic portion and did not decrease in the time course of ischemia.

Ca\(^{2+}\)-stimulated ATPase activity; Fig. 3 shows Ca\(^{2+}\)-stimulated ATPase activity of the SR fraction. Ca\(^{2+}\)-ATPase activity ranged 8.6 ± 2.1 μmoles/g wet weight/hr in Endo and 10.8 ± 2.3 in Epi in the non-ischemic portions. Twenty min after coronary occlusion, the activity reduced significantly to 66% of the control activity in Endo (p < 0.05), but the activity was kept at the control level in Epi. At 30 min, the activity decreased significantly to 31% (p < 0.01) and 58% (p < 0.01) of those in the non-ischemic portion in Endo and Epi, respectively, and no further decrease was observed until 120 min.

Gel electrophoresis of SR proteins; Electrophoretic gels of the cardiac SR are shown in Fig. 4. SR proteins were separated into the major ATPase protein (molecular weight of about 100,000 daltons), several acidic proteins (70,000–50,000), and proteolipids (12,000–6,000), as described in previous articles\(^5,\)\(^,\)\(^17\) A decrease in the band of the major ATPase protein was observed in the ischemic protein after 30 min of ischemia in both of Endo and Epi, and the bands of other proteins were not changed.
Fig. 5. Compositions of the major ATPase protein of the SR. Bars indicate mean ± SD. The open circles and the closed circles indicate the compositions of Endo and Epi, respectively. *p < 0.05, **p < 0.01 against control value.

Fig. 6. Electrophoretic gels of the SR from the non-ischemic myocardium incubated at a series of pH from 4.0 to 8.0. (10% acrylamide)

Fig. 7. Compositions of the major ATPase protein of the SR incubated at series of pH from 4.0 to 8.0. Bars indicate mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 against control value.
significantly.

The compositions of SR proteins; The compositions of SR proteins are given in Fig. 5. In the non-ischemic heart muscle the major ATPase protein occupied about 40% of total SR proteins. In the ischemic myocardium, composition of the major ATPase protein did not change until 20 min after coronary ligation, but at 30 min it decreased significantly to 55% (p < 0.01) and to 74% (p < 0.05) of the control values in Endo and Epi, respectively. For 60 min the content decreased continuously, but after 120 min of ischemia they were constant at the same low level as that of ischemia at 60 min.

Gel electrophoresis of the SR incubated at different pH; Gel electrophoresis of the SR incubated at a series of pH from 4.0 to 8.0 at 37°C are shown in Fig. 6. A similar pattern of

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change to that of in vivo ischemia was observed. The band of the major ATPase protein decreased significantly at a pH of 6.0 and 5.0.

Compositions of the major ATPase protein of the SR incubated at different pH; Compositions of the major ATPase protein of the SR incubated at different pH are shown in Fig. 7. The greatest decrease in the major ATPase protein was noted at pH 6.0 to 67% of that of the control SR. At the neutral or highly acidic state, the reduction in the major ATPase protein was much smaller than that at pH 6.0.

Activity of the cathepsin B in the SR and Ly fractions; Activity of cathepsin B in the SR and Ly fractions are shown in Fig. 8. In the SR obtained from non-ischemic heart muscle, cathepsin B activity ranged 6.04 ± 1.15 nmole naphthylamine/g wet weight/hr in Endo and 6.16 ± 1.38 in Epi at pH 7.0. In the ischemic portion, cathepsin B activity of both fractions did not change significantly at pH 7.0, however it increased markedly at pH 6.5 to about 170% of that at pH 7.0 in the non-ischemic portion from 10 to 30 min after ligation, and decreased in both fractions obtained from Endo after 60 min, indicating leakage of the enzyme to the cytosol.

Effect of pH change on activity of cathepsin B; Fig. 9 presents the effect of pH change on cathepsin B activity in the SR and Ly fractions. The maximal activity noted at pH 6.5, was about 160% of the activity at pH 7.0 in both the SR and Ly fractions, and the activity decreased in the acidic state under pH 5.5 or alkaline state over 7.0, exhibiting pH dependency.

DISCUSSION

The SR has been established to be the site of excitation-contraction coupling in the cardiac muscle by transporting Ca ions, and Ca-stimulated-ATPase of the SR is known to work as a Ca pump in the uptake of cytosolic Ca ions to the inside of the SR utilizing energy. In the ischemic myocardium disturbance of Ca transport has been described in many articles. Lee et al. revealed a decrease in the rate of Ca-uptake after coronary occlusion for 60 to 90 min. Hess et al. reported that the reduction in the Ca-uptake rate after 20 to 30 min of ischemia in Endo and Epi, and a significant decrease in Ca-ATPase activity was noted in ischemia for 30 min in Endo. In a cytochemical electron microscopic study, the reduction in Ca-ATPase activity in the terminal cisternae of the SR was observed accompanied with fine structural ischemic changes after coronary ligation for 30 and 60 min. The recent study demonstrated that intermembranar particles of the SR, most of which were thought to be the Ca-ATPase protein itself, decreased in ischemia for 30 to 60 min with a reduction in both Ca-ATPase activity and the content of the major ATPase protein. Toba et al. noted the degradation of the major ATPase protein simultaneously with decreases in Ca-uptake and Ca-ATPase activity in ischemia for 60 to 180 min.

In the present study which divided the myocardium into Endo and Epi, we observed that Ca-ATPase activity began to decrease after 20 min of ischemia in Endo and 30 min in Epi in a similar manner to those reported in previous reports. Furthermore, it was demonstrated that a decrease in the composition of the major ATPase protein occurred earlier than the time which Toba et al. described—as early as 20–30 min after coronary ligation in Endo and at 30 min in Epi. It is likely that reduction in Ca-ATPase activity occurred earlier than degradation.
of major ATPase protein in ischemia for 30 min in Endo, but we presume the noncongruity of the data is caused by the delicately dispersed results obtained at a critical point of ischemic myocardial injury, or by tiny undetectable alterations in SR proteins, such as the conformation change of the ATPase protein.

Since ATPase protein is the most essential component of the SR membrane, and its degradation means not only disturbance of its function but also destruction of the SR membrane substructures itself, it should be said the decrease in the contents of the major ATPase protein is an important indication of irreversible ischemic changes. Consequently the results of this study suggest the irreversible change begins earlier in ischemia for 20 to 30 min in Endo, but extends to Epi at a later time.

Up to this point, evidence of irreversible changes in ischemic myocardium has been described mainly by morphologic studies. Jennings et al. showed irreversible damage, such as swollen mitochondria containing amorphous matrix densities in ischemia for 40 to 60 min. Those changes were exhibited to progress from Endo to Epi, and it was termed “the wavefront phenomenon.” In the present study, we also demonstrated that the SR membrane degrades earlier in Endo than it does in Epi, supporting their hypothesis. However in our observation, degradation of the SR membrane occurred earlier than that in the report of Jennings et al., i.e. 20 to 30 min in Endo. This indicates that irreversible injury of the SR membrane does occur earlier than the appearance of the morphologic changes.

Several factors have been discussed regarding the initiation of ischemic myocardial injury and necrosis such as a lowered ATP content, accumulation of intermediate metabolites in the anaerobic metabolism, pH reduction, as discussed in this article, and the excess inflow of Ca ions according to the alterations in membrane permeability. We also have investigated degradation of membranes composing phospholipids in the experimental system, and found the degradation of phosphatidylcholine and phosphatidylethanolamine in the SR and mitochondrial fractions at around 30 to 60 min after the coronary ligation. Now we believe firmly that the activation of phospholipase C originates these changes in membranes composing phospholipids by the excess inflow of Ca ions. These activities seem to be sound in their own way, and many such simultaneous reactions may be generated at one time in the very early ischemic myocardium, resulting in the initiation of ischemic myocardial necrosis.

The activation of acid hydrolases inside of the SR has been considered particularly instrumental in the degradation of the SR proteins. Since the demonstration by De Duve and Beaufay that the acid hydrolases, which were located inside of lysosomes, were activated in the ischemic liver and were greatly concerned with the hepatic death, such a mechanism has been investigated in case of ischemic myocardial injury. As discussed above, the presence and activation of acid hydrolases in both lysosomes and SR were noticed by Hoffstein and associates in the process of ischemic myocardial injury, and these were also supported by biochemical and electron microscopic studies. As a matter of fact, in the initial stage of myocardial ischemia in anaerobic metabolism, intracellular pH is reduced to about 6.0 and the activation of lysosomal acid hydrolases should cause irreversible myocardial damage by digesting the intracellular micro-organisms consequently. This speculation was advocated by Wildenthal et al. and named “lyosomal hypothesis.”

In in vitro study, i.e. incubation of the isolated SR fraction in the acidic state, the maximal degradation of ATPase protein was noted at a pH of 6.0. This phenomenon indicates that the ATPase protein is digested by the protease existing inside of the SR, wherein the optimal pH should be about 6.0. From this point of view, we have focused attention on cathepsin B, which is one of the well known lysosomal endopeptidases. The activity of cathepsin B on many native proteins was shown in recent studies and its optimal activity was close to a pH of 6.0 with most substances. In the present study, the optimal activity was observed at pH 6.0–6.5 in both of the Ly and SR fractions, with respect to cathepsin B, and exhibited pH dependency. In the ischemic heart muscle, the activity of cathepsin B in both of the Ly and SR fractions was markedly higher at a pH of 6.5 than that at 7.0 in the non-ischemic portion after coronary ligation for 10 to 30 min. These results suggest that cathepsin B is activated not only in lysosomes but also in the SR in lowered intracellular pH in the early stage of ischemic myocardium.

Based on this evidence of activation of cathepsin B in the SR and the degradation of
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ATPase protein, it is considered that cathepsin B plays an initial role on the degradation of the SR membrane (among many other factors related to the degradation of the ischemic myocardium), and its action is direct digestion of the ATPase protein itself.

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