IMPAIRMENT OF SARCOLEMMAL PERMEABILITY IN THE ACUTE ISCHEMIC MYOCARDIUM

— A Study with Ionic Lanthanum Probe Method —

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Impairment of sarcolemmal permeability after the initiation of acute ischemic myocardial injury was studied using an ionic lanthanum (La^{3+}) probe in electron microscopy. Acute ischemic myocardium was induced by ligation of the left anterior descending coronary artery in dogs.

In normal cardiac myocytes La is localized exclusively in the extracellular space, i.e. on the glycocalyx, in the T-system and in intercalated discs, with normal fine structures. In ischemic myocardial cells, La deposits were found in the cytosolic space in 22% of subendocardial cells with mild to moderate, but non-necrotic, fine structural changes as early as 30 min. The number of myocytes with La deposition increased with advancing ischemic injury, and necrotic fine structural changes were recognized following ischemia for 60 min.

These results indicate that deposition of La occurs before the appearance of irreversible morphologic alterations in ischemic myocardial cells, and suggest an increased permeability of the sarcolemma for Ca^{2+} and the development of degradation of plasma membrane integrity. (*Jpn Circ J* 1992; **56**: 911–920)

ALTERED membrane integrity was strongly suggested as one of the major factors in the initiation of irreversible cellular injury during myocardial ischemia¹. However, the cellular mechanisms with which myocardial ischemia induces irreversible injury and cell death have not yet been clearly defined.

Irreversible ischemic injury has been noted in electron microscopy as early as 40 to 60 min² after the cease of coronary blood flow. So-called mitochondrial dense deposits appear with severely disorganized and

ruptured cristae. The nature of these granules was elucidated using microincineration and X-ray microanalysis to be the accumulation of Ca^{2+} and denatured protein^{2,3}. In addition to biochemical studies, much evidence suggests the uncontrolled influx of Ca^{2+} from the extracellular space^{4–6}. An early event in the pathogenesis of ischemic injury seems likely to be damage to the sarcolemma, causing increased permeability to metal ions before clear fine structural changes are recognized⁷. Langer and the co-workers^{8,9} suggested that the ability of a cation to displace surface-bound Ca^{2+} and uncouple excitation from contraction depends on the size of the non-hydrated ionic radius, and described the possibility of using

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Fig.1. Deposition of La in ischemic myocardial tissue.

Ischemic myocardial tissue was immersed in a solution containing 4 mM LaCl_3 . Cardiac myocytes with La deposition were counted. Fifty myocytes were counted in each period of ischemia. Open circle: ischemia for 180 min, closed circle for 60 min, and X for 30 min, respectively. A longer incubation period than 15 min induced serious autolytic changes, therefore, 10 min of incubation was adopted.

La^{3+} as a tracer to demonstrate altered permeability of the plasma membrane. Ultrastructural examination of cardiac muscle cells reveals fine extracellular precipitates detectable on sections by using ionic lanthanum^{8,9}. Several ultrastructural studies have shown that La selectively binds to the sarcolemma and is not visualized ultrastructurally within normal cardiac muscle cells⁸⁻¹¹.

The present study was undertaken to detect uncontrolled inflow of extracellular Ca^{2+} as a result of increased permeability of the sarcolemma in the early stage of ischemic myocardial injury. It used the ionic lanthanum probe technique in experimental acute ischemic myocardial cells in the dog.

MATERIALS AND METHODS

Animal experiment: Fifty adult mongrel dogs weighing 8 to 14 kg were anesthetized by intravenous injection of sodium pentobarbital (Nembutal®) at 30 mg/kg body weight. A left thoracotomy was carried out under a positive pressure respiration

using a Harvard type respirator, and the left anterior descending coronary artery (LAD) was excised and totally occluded with a silk thread just below the first diagonal branch.

Electrocardiogram was recorded and the occurrence of severe transmural ischemia was confirmed by continuous ST segment elevation of greater than 0.5 mV in the leads V_3 and V_4 to 180 min after LAD ligation. Four sham-operated dogs were similarly prepared that without LAD ligation.

At 30, 60 and 180 min after coronary ligation, the beating heart was removed and washed briefly with ice-cold saline solution. Eight to nine dogs were utilized in each ischemic period. The central portion of ischemic subendocardial muscle was excised from the left ventricular anterior wall. A portion from the posterior wall was excised as a non-ischemic control.

Cytochemical lanthanum reaction: A myocardial sample was immediately minced to 1 mm³ with a razor blade and incubated with a solution containing 4 mM LaCl_3 , 8% sucrose and 0.1 M Na-cacodylate (pH 7.4) at room temperature for 10 min. The composition of the reaction medium was modified

Fig.2. A normal myocardial cell.

Fine structures are preserved intact, and La deposition was localized in the extracellular space. Gl; glycocalyx, T; T-tubular system, Mt; mitochondria, and ICD; intercalated disc. A bar indicates $1\mu\text{m}$.

Fig.3. A myocardial cell from the non-ischemic portion of an ischemic heart.

Fine structures and La deposit are similar to those of a normal myocyte. Gl; glycocalyx, and Mt; mitochondria. A bar indicates $1\mu\text{m}$.

Fig.4. An ischemic myocardial cell for 30 min.

Slight swelling of mitochondria and slight intracellular edema are observed. La deposition is observed in the cytosolic space, on myofilaments and around mitochondria. Gl; glycocalyx, and Mt; mitochondria. A bar indicates 1 μ m.

from that of Martinez-Palomo et al.¹¹ Incubation period was checked from 5 to 60 min by observing 50 myocytes in each incubation time in each period of ischemia. With longer incubation periods, La was observed deep inside the myocardial tissue with serious autolytic cellular changes. Finally, 10 min of incubation period was employed with minimal or almost no autolytic morphologic changes and with sufficient permeation of La in ischemic myocardial cells (Fig. 1). They were fixed in 2% glutaraldehyde and 0.1 M Na-cacodylate (pH 7.4) at 4°C for 60 min, rinsed and postfixated in 1% OsO₄ at 4°C for 60 min, dehydrated in a graded series of ethanols, and embedded in Epon 812. Ultra-thin sections (60–70 nm thick) were cut on a Porter Blum MT-2, mounted on copper grids and double stained. Sections were observed with a Hitachi H-300 transmission electron microscope.

Cell counts were carried out to semi-quantitate ischemic myocellular injury according to the classification by Ozawa et al.¹² i.e. normal and slightly injured, moderately injured, and severely injured

cells. Moderate structural injury was characterized by slight intracellular edema, decrease in glycogen granules, slightly swollen mitochondria with reduced matrix density and prominent clumping of nuclear chromatin.

Severe structural damage was characterized by marked intracellular edema, disarray of myofibrils, intramitochondrial dense deposit and sarcolemmal disruption. Cells containing La deposits were also counted independently of cellular injury. A total of 300 cells by random selection were examined in each period of ischemia.

X-ray microanalysis: Analytical electron microscopy was utilized with energy dispersive X-ray detector (Kevex) to identify the electron dense deposits composed of La.

RESULTS

Normal and non-ischemic cardiomyocytes; Fig. 2 and 3 show normal and non-ischemic myocardial cells, respectively. Normal myocardial cells from the sham-operated dogs as controls show normal ultrastructure. La was observed only in the extracellular

Fig. 5. A myocardial cell from ischemic myocardium for 60 min. Intramitochondrial dense deposits are recognized with severe intracellular edema. Marked deposition of La inside the cell is seen. Gl; glycocalyx, Mt; mitochondria, and DD; dense deposit. A bar indicates 1 μ m.

spaces, i.e. on the glycocalyx, in the T-system, and in intercalated discs, but not inside the myocytes (Fig. 2). Non-ischemic myocardial cells from the posterior wall of the left ventricle exhibited similar features and were used as controls in each experiment (Fig. 3).

Ischemic myocardial cells; After ischemia for 30 min, slight to moderate La deposits were found inside myocytes. La deposition was observed in 22% of cells on myofibrils and around mitochondria. These cells showed slight to moderate ischemic injury, i.e. slight intracellular edema and swollen mitochondria with irregular cristae,

but fine structures were relatively well preserved (Fig. 4). These cells were assumed to be still salvageable with reconstruction of coronary blood flow.

After ischemia for 60 min, cells with severe injury appeared (Fig. 5). La deposits increased markedly in the myocytes and were distributed mainly on myofibrils and around mitochondria. Intramitochondrial dense deposits were noted. Prominent clumping of nuclear chromatin was recognized with moderate to severe cytoplasmic and mitochondrial edema. Disruption of swollen sarcoplasmic reticulum and disarray of myofibrils were noted in places.

Fig.6. Ischemic fine structural changes and La deposit in ischemic myocardial cells. Percentage of myocytes in which La accumulated is shown on the left side bar, and fine structural changes on the right side after the same ischemic period. At 30 min of ischemia, La depositions were observed preceding the appearance of severe fine structural changes, and the ratio of La accumulated myocytes increased in longer ischemia.

Fig.7. Correlation of La deposition and fine structural changes. Percentages of three grades of morphologic changes in myocytes with La deposits (+) and those without (-), respectively. Fine structural alterations are more frequent and were severe in ischemic cardiac myocytes containing La deposition.

After ischemia for up to 180 min these cells with advancing ultrastructural changes increased in number, as did cells with La

deposition.

In the ischemic myocardium, cellular injury was non-homogeneous. There was a

Fig.8. X-ray microanalysis of electron dense deposition after reaction with La^{3+} . Deposit on glycocalyx (8 A-a) and that on myofilament (8 A-b) contains La as shown in Fig. 8B and 8C, respectively. However, cytoplasm (8 A-c) does not contain deposit as shown in Fig. 8D. SL; sarcolemma, Mf; myofibril.

mixture of cells with various grades of injury. To semi-quantitate the grade of injury, cell-counting was carried out as described above. Fig. 6 shows the ischemic fine structural alterations as classified in our previous report by Ozawa and co-workers¹² and intracellular deposition of La.

In non-ischemic myocardial cells, fine structures were well-preserved or only slightly damaged. No La deposition was found inside the myocytes.

After ischemia for 30 min, cells with various grades of injury appeared. Normally shaped or slightly injured cells accounted for 56% of the total; 36% of cells had moderate injury and were considered to be still viable; only 8% of cells showed severe ischemic injury. La deposits were found inside about 22% of cardiac myocytes.

After ischemia for 60 min, intracellular La

deposition increased with an increasing number of cells with moderate to severe injury.

After 180 min of ischemia, intracellular La deposition was found in 56% of cells, and 72% of cells had severe fine structural injury. The number of myocytes containing La deposition increased with advancing period of ischemia prior to the progression of the degenerative changes.

Fig. 7 shows grading of ischemic myocardial injury and intracellular La deposition. One hundred myocytes with or without La deposition were counted in each period of ischemia. This indicates that cells not containing La deposition were almost normal or slightly injured. In contrast, those containing La deposits had moderate to severe injury in each period of ischemia. The longer the ischemic period, the greater the number of severely injured cells with La

deposition.

X-ray microanalysis; Fig. 8 shows X-ray microanalysis of a myocardial cell in ischemia for 60 min. Analytical electron microscopy revealed a prominent La peak (a 4.65 keV peak) in the electron dense deposits on the glycocalyx (Fig. 8A-a and Fig. 8B), and on myofibrils (Fig. 8A-b and Fig. 8C).

However, in a cytosolic portion no La peak was noted without dense deposition (Fig. 8A-c and Fig. 8D).

DISCUSSION

Great attention has been paid to the time dependent changes and cellular mechanisms of ischemic myocardial cellular injury to necrosis. Of particular interest is the efficacy of coronary intervention such as intracoronary thrombolysis (ICT) and protection from ischemic myocardial injury.

Calcium ions, which play one of the most important roles in the contraction and relaxation cycle of cardiac muscle¹³ have been reported to be significant triggers of the initiation of ischemic myocardial injury⁴⁻⁶. The appearance of a dense deposit in the mitochondria of ischemic myocardial cells was noted to be an irreversible morphologic signs as early as 40 to 60 min after the cease of coronary blood flow². The deposit was noted to contain Ca^{2+} , especially in the spicular form^{2,3}. Many biochemical studies have suggested an increase in cytosolic Ca^{2+} in the extra-cellular space in the early ischemic myocardium⁴⁻⁶. A four-fold increase in cytosolic Ca^{2+} was detected using ^{19}F -NMR in early ischemia before the appearance of ischemic fine structural alterations¹⁴. Calcium ion overload of cardiac myocytes was established to induce dramatic necrotic changes in perfusion experiment. The pathophysiology was found to be very similar to reperfusion injury of the ischemic heart muscle¹⁵.

In the early stages of myocardial ischemia, impairment of membranous microorganelles was described from our laboratory¹⁶⁻¹⁹. Akiyama¹⁶ and Konno¹⁷ described degradation of the major ATPase protein of the microsome fraction (mainly sarcoplasmic reticulum) after as little as 20 min of ischemia in the canine heart. Degradation of microsomal and mitochondrial membranes

was also noted by Yanagishita et al¹⁸ from the standpoint of membrane-constituting phospholipids at the same time as the degradation of protein components. Irreversible impairment of mitochondrial respiratory activity was also noted by Geshi and the co-worker¹⁹ after as little as 30 min of ischemia in the subendocardial muscle. The degradation of membrane-constituting phospholipids indicates the activation of phospholipases which need Ca^{2+} in their activation process. Observation of such alterations in the distribution of Ca^{2+} by electron microscopy is very difficult in ischemic myocardial cells. Takeyama and the co-workers²⁰ observed intramyocellular localization of metal ions, especially Ca^{2+} , in ischemic cells. They used a pyroantimonate probe method and found that intracellular distribution of Ca^{2+} , which flows from the extracellular space through impaired sarcolemma, corresponded well to fine structural alterations in ischemic cardiac myocytes. These biochemical and morphologic changes suggest that intramyocellular inflow of Ca^{2+} in early myocardial ischemia induces ischemic cellular injury, even though increased Ca^{2+} -influx was not so clearly detected as reoxygenation of hypoxic heart⁵.

Lanthanum has long been considered to have chemical properties similar to the alkaline earth metals. Recent interest in the biological action of La^{3+} is based on its being a substitute or antagonist for Ca^{2+} in a variety of cellular and subcellular reactions^{8,9,21,22}. The superficially located Ca^{2+} binding sites in cardiac muscle appear to be affected by lack of oxygen. Naylor and co-workers²³ reported that the amount of Ca^{2+} stored at membrane-located sites is reduced in ischemic or hypoxic muscle. Langer and co-workers^{8,9,22} studied the ability of sarcolemmal membranes to bind Ca^{2+} and concluded that the calcium displaceable from whole cells by lanthanum is located in sarcolemmal sites. In further studies they concluded that La^{3+} binds to a mucopolysaccharide-protein complex, the so-called glycocalyx of the sarcolemma.

Lanthanum was used as a tracer for Ca^{2+} on the cell surface and for the detection of impairment of sarcolemmal permeability. It is well known that in normal cardiac myocytes La deposition is confined to extracellular spaces, making no entry into the

cell cytoplasm. Hoffstein and associates⁷ noted the deposition of colloidal La (average diameter 40Å) in ischemic dog myocardial cells 3.5 h after the cease of coronary blood flow, and concluded that an increase in sarcolemmal permeability and loss of sarcolemmal integrity induced ischemic irreversible injury. A similar study was performed by Bloor and Ashraf²⁴ with ischemia for 45 min.

In the present study, ionic lanthanum was used as a tracer for Ca^{2+} to investigate loss of sarcolemmal integrity and the resulting uncontrolled Ca^{2+} influx which can trigger irreversible injury in the early stages of severe myocardial ischemia. La^{3+} is of a similar molecular size to Ca^{2+} .²¹ In the ischemic myocardium, La deposition in myocytes was observed after as little as 30 min of ischemia. However, most cardiac cells were ultrastructurally preserved intact or only slightly damaged. With a progression of the ischemic period from 30 to 180 min, myocellular fine structures were altered progressively and the number of cells containing intracellular La deposition increased. This evidence indicates that Ca^{2+} , as represented by La, flows into ischemic myocardial cells leading to the appearance of ischemic injury. There is a critical point of no-return, at around 30 min of ischemia, due to increased sarcolemmal permeability, i.e. loss of integrity, which acts as a trigger for the initiation of irreversible injury. Deposition of La was not observed in severely injured cells. It seems likely that La^{3+} did not penetrate into the central portion of soaked muscle tissue. Similar results were described by Burton and co-workers²⁵ utilizing the ionic lanthanum probe method in hypoxia and reoxygenation of feline papillary muscle in hypoxia for 1 h and 15 min. An increase in permeability of the sarcolemma is likely to be induced by such changes in early ischemic metabolism as reductions in tissue content of ATP and pH, and an inverse increase in H^+ . Ca^{2+} which flows into ischemic myocardial cells with increased membrane permeability is likely to activate several hydrolytic enzymes such as phospholipases¹⁸ and proteases, and also activate Ca^{2+} dependent pathways to waste energy.⁶ A series of these reactions is likely to lead the ischemic cardiomyocytes

to necrosis.

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