Cytoskeletal Damage During Myocardial Ischemia: Changes in Vinculin Immunofluorescence Staining During Total in Vitro Ischemia in Canine Heart

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Plasma membrane disintegration is a feature of lethal myocardial ischemia. Heart-specific proteins such as MB-CPK and cardiac myosin light chains appear in the systemic circulation, and circulating anti-myosin antibodies begin to accumulate in ischemic myocytes several hours after coronary artery occlusion in experimental animals and in patients undergoing myocardial infarction. Furthermore, studies of temporary ischemia and reperfusion have demonstrated a prompt washout of sarcoplasmic proteins during reperfusion after 1 hour of ischemia. These results indicate that ischemia promotes structural damage to myocyte plasma membranes. It is unclear whether this is an early or late event relative to the transition from reversible to irreversible injury, and it is unclear what role reperfusion may play in plasma membrane disruption during temporary ischemia followed by reperfusion.

Evidence that plasma membrane disruption occurs early in the irreversible phase of myocardial ischemia and does not require reperfusion to become manifest is provided by ultrastructural evaluation of subendocardial tissue that had been permanently ischemic for 40 minutes in vivo. This duration of severe in vivo ischemia is sufficient to produce lethal injury since a subendocardial infarct always is observed several days later, with or without reperfusion of the tissue with arterial blood. Electron micrographs of the permanently ischemic myocardium show breaks in the plasmalemma of the sarcoplemma while the basal lamina generally remains continuous. These ultrastructural alterations have been associated with release of intracellular proteins and entry of normally impermeant extracellular markers. Similar ultrastructural changes occur during total ischemia in vitro, although the time course is slower in vitro than in vivo. These observations suggest that the intracellular milieu that exists in ischemic myocytes is capable of promoting plasma membrane disruption without any requirement for exogenous agents such as oxygen or inflammatory cells.

In previous studies from this laboratory, we have explored the possibilities that phospholipid degradation and cell swelling contribute to plasma membrane disruption during total ischemia in vitro. Although production of lysophospholipids and arachidonic acid has been shown to occur early during oxygen deprivation, the rate of net phospholipid degradation...
does not appear to be sufficient to account for plasma membrane disruption during total ischemia. Studies on the effect of cell swelling on plasma membrane integrity suggested that cell swelling might be a factor in plasma membrane disruption but that a preceding period of oxygen and substrate deprivation was required to increase membrane fragility. It was also proposed that damage to the cytoskeleton might be the cause of the increased membrane fragility but no data were provided to support this hypothesis other than the observation that the breaks in the plasma membrane generally were associated with subsarcolemmal blebs where the cytoskeletal Z-line attachments between plasma membrane and the underlying myofibrils were broken. Previous studies have shown that the cytoskeletal protein vinculin is located at the Z-line attachments of the plasma membrane, as well as at t-tubular invaginations and intercalated disk regions in ventricular myocytes. Because of its location and its possible role in the linkage between the cytoskeleton and the plasma membrane, we chose to investigate the possibility that vinculin distribution might be altered during ischemia with a similar time course to the development of subsarcolemmal blebs and plasma membrane disruption. Vinculin was identified in tissue sections by immunofluorescence using an anti-vinculin primary antibody and a fluorescein-labelled secondary antibody. The results confirm the previously reported costameric (rib-like) pattern of fluorescence along the lateral margin of control myocytes and myocytes that were subjected to brief durations of ischemia and that retain intact plasma membranes. However, when the duration of total ischemia is long enough to be associated with rupture of the plasma membrane, there is a marked loss of anti-vinculin antibody staining. This suggests that cytoskeletal alterations may contribute to the spontaneous disruption of plasma membranes during myocardial ischemia.

Materials and Methods

Healthy adult mongrel dogs of either sex were anesthetized with intravenous sodium pentobarbital. They were subsequently intubated and ventilated with room air. The heart was excised through a left thoracotomy and immersed in 750 ml of ice-cold isotonic potassium chloride. Portions of one left ventricular papillary muscle were placed in a sealed container in a 37°C water bath, and the other left ventricular papillary muscle was used to prepare thin slices for incubation.

Slice Incubation Protocols

Thin slices of myocardium were prepared as described elsewhere. The slices were cut freehand and were approximately 0.5 mm thick and weighed between 30 and 100 mg (wet weight). Slices were placed in 25 ml Erlenmeyer flasks, generally 6 slices in 15 ml of media. The flasks and media were prewarmed in a shaker bath at 37°C and preequilibrated with O₂. The flasks were shaken 180 times/min in the 37°C shaker bath and were continuously equilibrated with humidified O₂. The basic medium was a Krebs-Ringer phosphate (KRP) solution containing 30 mM mannitol, 1% bovine serum albumin, and in some experiments, trace amounts of [¹⁴C] hydroxymethyl inulin.

Inulin Diffusible Space

In the experiments where inulin space measurements were made, tissue slices were removed from the flasks at the end of the period of incubation, dipped briefly in 0.25 M sucrose to remove excess KRP and inulin from the surface of the slices, blotted on Whatman number 1 filter paper, and weighed quickly on a Mettler AE100 microbalance. Subsequently, the slices were placed in an oven at 105°C overnight and reweighed to determine the dry weight and total tissue water of each slice. Dried slices were rehydrated with 2 drops of deionized water. The tissue was solubilized with 1 ml of Soluene 350 (Packard). The extracellular [¹⁴C] activity was determined by addition of 0.1 ml of the medium from each incubation flask to scintillation vials. The media vials were prepared in duplicate and were treated the same as the tissue vials, beginning with the addition of Soluene. Once the tissue samples were fully dissolved, 15 ml of acidified Instagel (Packard), prepared by adding 0.5 N HCl to Instagel in a ratio of 1:9, were added to each vial. The vials were counted in a Packard model 3255 liquid scintillation counter. The inulin diffusible space was calculated as described previously.

Vinculin Immunofluorescence

Tissue slices were embedded in gelatin and snap-frozen in isopentane cooled in liquid nitrogen. Frozen sections were cut at approximately 3–4 μm and were placed on gelatin-coated slides. In preliminary experiments, it was found that briefly dipping the sections in acetone at −20°C gave slightly enhanced immunofluorescence contrast as compared to unfixed sections, and the acetone rinse was used prior to immunofluorescence staining in subsequent experiments. After the acetone rinse, sections were incubated at room temperature for 15 minutes in normal goat serum diluted 1:10 with phosphate buffered saline (PBS) to minimize nonspecific staining. Next, the sections were incubated for 30 minutes at room temperature in polyclonal rabbit anti-vinculin antibody diluted 1:20 with PBS. The rabbit antibodies were a gift from Marina Glukhova (All-Union Cardiology Research Center of the USSR, Moscow) and were raised against vinculin obtained from chicken gizzard. After briefly rinsing the sections with PBS, the sections were incubated in goat anti-rabbit IgG labelled with fluorescein isothiocyanate at a 1:40 dilution for 30 minutes. Control slides were prepared for each section by substituting normal rabbit serum for the anti-vinculin antibody and by omitting the rabbit antibody entirely. The immunofluorescence slices were screened to identify areas where the myocytes were sectioned longitudinally. Representative fields were photographed at 250× and 400×.
Electron Microscopy

Tissue was minced under 4% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 (osmolality approximately 650 mOsm). The tissue was fixed for at least 4 hours, postosmicated for 1 hour in 1% osmium tetroxide, dehydrated in a graded ethanol series, rinsed in propylene-oxide, and embedded in Epon 812. At least two blocks were embedded for each experimental condition. Semi-thin sections were stained with toluidine blue, and areas were selected for thin sections in which the myocytes were longitudinally oriented. Two grids consisting of 3–5 thin sections were prepared from each specimen. The grids were stained with uranyl acetate and lead citrate and examined with a JEOL JEM 100B electron microscope. The central portions of the thin sections were examined thoroughly, and low and high magnification photographs were taken to evaluate plasma membrane ultrastructure. The criteria for ultrastructural evaluation of plasma membranes are given elsewhere.6

Results

To demonstrate that a rabbit polyclonal antibody raised against chicken smooth muscle vinculin would cross-react with mammalian myocardial vinculin, sections of control canine myocardium were incubated with the polyclonal anti-vinculin antibody after blocking nonspecific binding sites. Subsequently, the sections were incubated with fluorescein-labelled anti-rabbit immunoglobulin. Myocytes stained in this manner showed prominent fluorescence at the intercalated disks and punctate staining along the plasma membrane at the sites of the plasma membrane attachments to the underlying myofibrils at each Z-line. This is the same distribution reported by others for vinculin in cardiac muscle,16 which has been termed costameric.14 Also, there was some staining along Z-lines, which has been attributed to vinculin in the t-tubule invaginations of the sarcolemma.4 The same pattern of vinculin immunofluorescence was observed in myocardial slices that were incubated for 300 minutes in oxygenated media (Figure 1A). Some of the Z-line and intercalated disk staining was nonspecific since substitution of normal rabbit serum for the anti-vinculin antibody did not completely abolish staining at these sites (Figure 1B). Nevertheless, the peripheral staining along the plasma membrane is specific for vinculin since it occurs only in the presence of anti-vinculin.
antibody, and it corresponds to the known distribution of vinculin in cardiac muscle.

Previous studies have indicated that plasma membranes fragment during severe ischemia in vivo\(^5\)\(^6\) and during total ischemia in vitro.\(^6\)\(^7\)\(^9\) The breaks in the plasma membrane occur primarily in areas where the sarcolemma has been lifted off the underlying myofibrils and is separated from the myofibrils by large subsarcolemmal blebs that extend over many sarcomeres. The presence of the subsarcolemmal blebs is objective evidence of loss of sarcolemmal-Z-line attachments and suggests that injury to cytoskeletal components such as vinculin at these attachment sites might be a factor in plasma membrane disruption during ischemia. For this reason, changes in vinculin distribution were evaluated after various durations of total ischemia in vitro. After 60 minutes of total ischemia, electron microscopy revealed intact plasma membranes, and when slices were prepared from this tissue and incubated for 60 minutes in oxygenated Krebs-Ringer containing trace amounts of \(^{14}\)C inulin, the myocytes excluded inulin, resynthesized ATP and creatine phosphate, retained potassium, and excluded calcium.\(^15\) The inulin diffusible space (IDS) was the same as in control slices incubated in the same manner (IDS of 0.91 ± 0.03 ml/g dry weight for control slices, \(n = 17\), vs. 0.91 ± 0.04 ml/g dry weight for slices of myocardium previously subjected to 60 minutes of total ischemia in vitro, \(n = 7\)). The pattern of vinculin staining after 60 minutes of total ischemia was essentially identical to that seen in control myocardium (Figure 2A), and as shown in Figure 2B, the pattern did not change noticeably during a subsequent 60-minute incubation in oxygenated medium, although the intensity of the staining appeared to be decreased.

After 120 minutes of total ischemia, changes associated with lethal injury were present. Electron micrographs revealed widespread breaks in plasma membranes although substantial portions of the plasma membrane of each myocyte were ultrastructurally intact. Representative ultrastructural changes observed after 120 minutes of total ischemia are shown in Figure 3. Two large subsarcolemmal blebs (Figure 3A) separating the sarcolemma from the underlying myofibrils are present while other portions of the sarcolemma have retained normal ultrastructure including Z-line attachments. The myocyte in the center of Figure 3A has a large bleb on one side and apparently intact

**FIGURE 2.** Vinculin immunofluorescence of left ventricular myocardium after 60 minutes of total ischemia in vitro (Panel A) and after 60 minutes of ischemia and 60 minutes of incubation in oxygenated media (Panel B). Representative longitudinal sections labelled with polyclonal antivinculin antibody are shown. (Magnification: 500 × )
FIGURE 3. Electron micrographs of left ventricular myocardium subjected to 120 minutes of total ischemia in vitro. A representative longitudinal section of several ischemic myocytes is shown in Panel A. Mitochondrial amorphous matrix densities (AMD) are apparent. Large subsarcolemmal blebs (SB) have separated sarcolemma (SL) from underlying myofibrils. Center myocyte has a large subsarcolemmal bleb along one (lower) lateral margin but ultrastructurally normal sarcolemma on the opposite (upper) side. Panel B shows a portion of ultrastructurally normal sarcolemma in greater detail than Panel A, and Panel C shows a portion of sarcolemma overlying a subsarcolemmal bleb. There are numerous breaks in plasmalemma (indicated by arrowheads) but glycocalyx (G) appears to be intact. (Magnification: A, 11,000×; B, 41,250×; C, 41,250×).
sarcolemma on the opposite side. This demonstrates the focal nature of the ultrastructural changes after 120 minutes of total in vitro ischemia. Figure 3B shows a higher magnification of the sarcolemma in an area where the Z-line attachments have been preserved. The plasma membrane is intact. In contrast, the sarcolemma overlying a subsarcolemmal bleb (Figure 3C) has an intact glycocalyx, but the plasma membrane is fragmented and is focally replaced by membrane vesicles. These electron micrographs illustrate a characteristic feature of the altered architecture of the sarcolemma during ischemia: the close spatial correlation between subsarcolemmal bleb formation and plasma membrane disruption. This correlation exists after all durations of myocardial ischemia and suggests that these two ultrastructural changes may be related.

Evaluation of vinculin localization in ischemic myocardium with focal ultrastructural lesions in the sarcolemma showed a heterogeneous pattern with areas of preserved costameric staining and other areas of sarcolemma which were devoid of staining (Figure 4A, 120 minutes of total ischemia in vitro). Incubation of slices prepared from myocardium that had been ischemic for 120 minutes, in oxygenated media, results in a small amount of creatine phosphate and ATP resynthesis but also substantial loss of magnesium, potassium, and creatine and marked calcium uptake, indicating that most of the ischemic myocytes were lethally injured. The IDS increased to $1.63 \pm 0.15$ ml/g dry weight ($n = 7$), and electron microscopy revealed a greater degree of plasma membrane disruption than was present prior to incubation. Figure 4B shows that the proportion of sarcolemma with punctate vinculin immunofluorescence also is reduced further during incubation as compared with the unincubated vinculin immunofluorescence (Figure 5), which, because it was absent, did not decrease further during incubation of the slices. This correlated with the ultrastructural appearance of generalized plasma membrane disruption in the permanently ischemic tissue and the marked increase in IDS to $2.44 \pm 0.06$ ml/g dry weight ($n = 8$) when slices of this tissue were incubated for 60 minutes in oxygenated inulin-containing media.

**Discussion**

The results demonstrate that there is a close relation between vinculin staining along the lateral margin of myocytes and plasma membrane ultrastructure during ischemia. The persistent, faint Z-line and intercalated disk staining can be attributed to non-specific staining as shown in Figure 1B. (Magnification: 500 x)
total ischemia in vitro. When the duration of ischemia is shorter than that required to produce plasma membrane disruption, i.e., 60 minutes in vitro, the pattern of vinculin immunofluorescence is preserved. Furthermore, incubation of tissue slices prepared from tissue that had been ischemic for 60 minutes, in oxygenated media, altered neither plasma membrane ultrastructure nor the distribution of vinculin staining. With the advent of sarcolemmal disruption induced by longer periods of total ischemia, there was concomitant loss of vinculin staining. Associated with this change was a marked increase in the inulin-diffusible space of thin slices prepared from this tissue as well as much less vinculin fluorescence in the slices. These data taken together support the hypothesis that disintegration of the cytoskeletal-plasmalemmal attachment complex may be the feature of ischemia that allows the plasma membrane to break as a consequence of the cell swelling that occurs in either severe or total ischemia.10,17

The changes in sarcolemmal integrity developed in totally ischemic tissue, i.e., quiescent tissue receiving no arterial collateral flow. It is clear, therefore, that sarcolemmal disruption in this system develops without any requirement for exogenous oxygen, water, calcium, inflammatory cells, platelets, or other bloodborne elements. Moreover, since the changes occurring in total ischemia in vitro are indistinguishable from those seen in low-flow ischemia in vivo, our observations emphasize that significant and substantial changes occur in the plasma membrane of the myocyte while it is ischemic.

The changes in vinculin distribution occurring in thin tissue slices prepared after varying intervals of ischemia and incubated in oxygenated Krebs-Ringer phosphate depend on the degree of injury present at the end of the ischemic period. No evidence of sarcolemmal damage is noted in tissue incubated after 60 minutes of ischemia. After 120 minutes of ischemia, changes were obvious but heterogeneous in the permanently ischemic tissue, and incubation appeared to exacerbate them. It is unclear whether this represents primarily accelerated destruction of myocytes that had focally disrupted plasma membranes at the end of the ischemic period, or whether myocytes that were structurally intact at the end of the ischemic period also are continuing to undergo changes associated with lethal damage. On the other hand, after 180 minutes of total ischemia, there was no detectable vinculin staining along the lateral margin of the ischemic myocytes; the changes were fully developed in the permanently ischemic tissue and were unaffected by subsequent slice incubation. These results, taken together, show that reoxygenation is not required for extensive disintegration of cytoskeletal components underlying the plasma membrane during myocardial ischemia.

Mechanisms of Cytoskeleton and Plasma Membrane Damage

The nature of cytoskeletal-plasma membrane attachments has been studied in various cell types and considerable data are available concerning the cytoskeletal components which are present at or near the attachment sites,14,18-20 but the precise role of these components and how they interact is less clear.21 The cytoskeleton presumably is responsible for maintenance of cell shape and plasma membrane integrity since the plasma membrane is an unstable structure that spontaneously forms small membrane vesicles if isolated or dissociated from the underlying cytoskeletal scaffold.22 Blebbing of epithelial cell plasma membranes is associated with dispersion of the microfilamentous cytoskeleton, which normally lies just beneath the plasma membrane and runs parallel to it.23 Thus, the
available evidence suggests that the cytoskeleton is essential for maintenance of plasma membrane integrity and that there is a close association between cytoskeletal damage and plasma membrane alterations. The precise role of vinculin in cytoskeleton–plasma membrane attachment is uncertain. Initial reports suggested that vinculin bound directly to actin, but subsequent data obtained with more highly purified vinculin suggest that vinculin is not an actin-binding protein. Although the precise arrangement of cytoskeletal proteins at the cytoskeleton-plasma membrane attachments is unclear, it appears that vinculin may serve as the final link to the membrane since it extends into the lipid bilayer of the plasmalemma.

One possible mechanism of cytoskeletal damage is proteolysis of cytoskeletal components. Calcium-dependent proteases have been identified in cardiac and skeletal muscles, which are capable of degrading many cytoskeletal proteins including vinculin. These proteases appear to be similar in both tissues and have been localized to Z-bands and on the cytoplasmic side of the plasma membrane. Two forms of the protease have been identified: one requires micromolar and the other millimolar levels of calcium for activation. In addition, each of the calcium-dependent proteases can autolyze, in which case the calcium requirement is decreased. The calcium concentration for half-maximal activity of the micromolar calcium-dependent protease is reduced from 10 μM to 2 μM following autolysis. In addition to regulation by calcium concentration and by autolysis, protease activity is modulated by an endogenous protein inhibitor. The precise method of regulation of the protein inhibitor–protease interaction is unclear. However, even though the protein inhibitor has been identified in platelets, cytoskeletal protein degradation occurs when cytosolic calcium rises to approximately 3 μM.

The present study shows that cytoskeletal damage occurs during myocardial ischemia and supports the hypothesis that the damage occurs because of activation of endogenous proteases by increased cytosolic free calcium. It has been shown that cytosolic calcium increases in energy-deficient myocytes, although the magnitude of the increase generally is small and the increase generally is delayed relative to the onset of inhibition of energy production. Nevertheless, the presence of micromolar calcium-dependent protease on the cytoplasmic side of the sarcolemma, the ability of the protease to degrade cytoskeletal proteins, and the observation that cytoskeletal proteins are degraded when the calcium concentration rises to 3 μM in stimulated platelets all are consistent with the hypothesis that proteolysis is responsible for cytoskeletal damage during ischemia. The cytoskeletal damage would weaken the plasma membrane and make it susceptible to rupture when stressed. The stress could be cell swelling or contracture, which would cause plasma membrane disruption while the tissue remained ischemic.

In summary, the present data indicate that cytoskeletal damage occurs in totally ischemic myocardium with a time course that parallels the development of plasma membrane disruption. These changes occur spontaneously during ischemia and do not require any exogenous mediators. The data emphasize the importance of events occurring while the tissue is ischemic, prior to reoxygenation or reperfusion.

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