Redistribution of phosphatidylethanolamine and phosphatidylserine precedes reperfusion-induced apoptosis

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Maulik, Nilanjana, Valerian E. Kagan, Vladimir A. Tyurin, and Dipak K. Das. Redistribution of phosphatidylethanolamine and phosphatidylserine precedes reperfusion-induced apoptosis. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H242–H248, 1998.—Although cardiomyocyte death and infarction associated with ischemia-reperfusion are traditionally believed to be induced via necrosis, recent studies implicated apoptotic cell death in ischemic reperfused tissue. To examine whether myocardial ischemic reperfusion injury is mediated by apoptotic cell death, isolated perfused rat hearts were subjected to 15 and 30 min of ischemia as well as 15 min of ischemia followed by 30, 90, or 120 min of reperfusion. At the end of each experiment, hearts were processed for the evaluation of apoptosis and DNA laddering. Apoptosis was studied by visualizing the apoptotic cardiomyocytes by direct fluorescence detection of digoxigenin-labeled genomic DNA using APOPTAG in situ apoptosis detection kit. DNA laddering was evaluated by subjecting the DNA obtained from cardiomyocytes to 1.8% agarose gel electrophoresis and photographed under ultraviolet illumination. In addition, high-performance thin-layer chromatography (HPTLC) of aminophospholipids labeled with 2,4,6-trinitrobenzenesulfonate was used to evaluate phospholipid topography in cardiomyocytes. The results of our study revealed apoptotic cells only in the 90- and 120-min reperfused hearts as demonstrated by the intense fluorescence of the immunostained digoxigenin-labeled genomic DNA when observed under fluorescence microscope. None of the ischemic hearts showed any evidence of apoptosis. These results corroborated with the findings of DNA fragmentation that showed increased ladders of DNA bands in the 120-min reperfused hearts, representing integer multiples of the internucleosomal DNA length (180 bp). Two-dimensional HPTLC of the phospholipids obtained from the cardiomyocytes and transbilayer organization of the phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the myocytes indicated translocation of both PE and PS from the inner leaflet to the outer leaflet of the plasma membrane, permitting tight packing of phospholipid head groups, whereas the inner leaflet has looser packing because of the presence of unsaturated fatty acids in the PE and PS at the outer leaflet of the cardiac membrane. DNA laddering was evaluated by subjecting the DNA obtained from cardiomyocytes to 1.8% agarose gel electrophoresis and photographed under ultraviolet illumination. In addition, high-performance thin-layer chromatography (HPTLC) of aminophospholipids labeled with 2,4,6-trinitrobenzenesulfonate was used to evaluate phospholipid topography in cardiomyocytes. The results of our study revealed apoptotic cells only in the 90- and 120-min reperfused hearts as demonstrated by the intense fluorescence of the immunostained digoxigenin-labeled genomic DNA when observed under fluorescence microscope. None of the ischemic hearts showed any evidence of apoptosis. These results corroborated with the findings of DNA fragmentation that showed increased ladders of DNA bands in the 120-min reperfused hearts, representing integer multiples of the internucleosomal DNA length (180 bp). Two-dimensional HPTLC of the phospholipids obtained from the cardiomyocytes and transbilayer organization of the phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the myocytes indicated translocation of both PE and PS from the inner leaflet to the outer leaflet of the plasma membrane as early as after 20 min of ischemia. These results demonstrate that the redistribution of PS and PE precedes the apoptotic cell death and DNA fragmentation associated with the reperfusion of ischemic myocardium, suggesting that ischemia may trigger the signal for apoptosis although it becomes evident during reperfusion.

Although the cardiomyocyte death and infarction associated with ischemia-reperfusion injury are traditionally believed to be induced via necrosis, which is a clear-cut mechanism of accidental cell death, its precise mechanism remains unclear. Recent studies implicated apoptotic cell death in ischemic brain (34) and ischemic liver (10). Reperfusion of ischemic renal tissues was found to be associated with apoptotic cell death (12, 32). Cardiomyocytes exposed to hypoxia revealed apoptotic cell death as evidenced by DNA fragmentation in conjunction with the expression of Fas mRNA (33). More recently, apoptotic and necrotic myocyte cell deaths associated with ischemia-reperfusion were shown to be independent contributing variables of infarct size in rats (17). A study from our laboratory also demonstrated apoptotic cell death in conjunction with myocardial ischemia and reperfusion (20). Another study has shown apoptosis to be a feature of human vascular pathology, including restenotic lesions and, to a lesser extent, atherosclerotic lesions, suggesting that apoptosis may modulate the cellularity of lesions that produce human vascular obstruction (15). Loss of membrane phospholipid asymmetry is one of the mechanisms leading to macrophage recognition of apoptotic cells. Decreased packing of membrane phospholipid head groups and cell shrinkage precede DNA fragmentation and may serve as early markers for the cells destined to become apoptotic (23). In normal cells, phosphatidylcholine and sphingomyelin containing saturated fatty acids dominate the outer leaflet of the plasma membrane, permitting tight packing of phospholipid head groups, whereas the inner leaflet has looser packing because of the presence of unsaturated fatty acids in phosphatidylserine (PS) and phosphatidylethanolamine (PE) (36). The reduction of aminophospholipid translocase resulting in PS translocation is believed to be the hallmark of apoptosis (11). It is generally believed that alterations in the fraction of PS in the outer leaflet of the plasma membrane reflect changes in the distribution of existing phospholipids rather than changes in its synthesis. Macrophages rapidly recognize PS on the apoptotic cells and readily remove them (8). A pathway by which apoptotic cells become targeted for macrophage scavenging is via aminophospholipid translocase. This enzyme is situated at the outer surface of the cell and might be one of the earliest enzymes to undergo oxidative damage induced by reperfusion.

Here we show that reperfusion, but not ischemia, induces apoptosis and DNA fragmentation. Dramatic changes in PE and PS redistribution precede the apoptotic cell death associated with the reperfusion of ischemic myocardium.

MATERIALS AND METHODS

Isolated rat heart preparation. Sprague-Dawley rats weighing ~300 g were anesthetized with pentobarbital sodium (80 mg/kg ip). After the rats received intravenous administration of heparin sodium (500 IU/kg), their chests were opened, and
the hearts were rapidly excised and mounted on a nonrecirculating Langendorff perfusion apparatus (21). Retrograde perfusion was established at a pressure of 100 cmH₂O with an oxygenated normothermic Krebs-Henseleit bicarbonate (KHB) buffer with the following ion concentrations (in mM): 118.0 NaCl, 24.0 NaHCO₃, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.7 CaCl₂, and 10.0 glucose. The KHB buffer had been previously equilibrated with 95% O₂-5% CO₂, pH 7.4 at 37°C. After perfusing the heart via the Langendorff mode for 10 min, hearts were made globally ischemic by terminating the coronary flow for 15 or 30 min. In a separate set of experiments, hearts were made ischemic for 15 min followed by 30, 90, or 120 min of reperfusion.

Evaluation for apoptosis. Apoptotic cell death was evaluated using the terminal deoxynucleotidyl transferase enzyme for nick end labeling (TUNEL) method (31) using APOPTAG kit (Oncor, Gaithesburg, MD). In brief, control, ischemic, and reperfused heart tissues were immediately put in 10% Formalin and fixed in an automatic tissue-fixing machine. The tissues were carefully embedded in the molten paraffin in metallic blocks, covered with flexible plastic moulds, and kept under freezing plates to allow the paraffin to solidify. The metallic containers were removed, and tissues became embedded in paraffin on the plastic moulds. Ten sections were made from each sample to make a clear judgment for each experiment. Before tissues were analyzed for apoptosis, tissue sections were deparaffinized with xylene and washed in succession with different concentrations of ethanol (absolute, 95%, and 70%). Tissues were then treated with proteinase K for 15 min at room temperature, excess liquids were carefully blotted around the sections, 1× equilibrium buffer was applied directly on the specimens, and specimens were placed in a humidified chamber for 5 min at room temperature. Specimens were then treated with terminal deoxynucleotidyl transferase at 37°C for 1 h in a humidified chamber. After 1 h, coverslips were removed, and the specimens were placed in a Coplin jar containing stop/wash buffer (supplied in kit) for 10 min at room temperature. Working strength antidigoxigenin fluorescence (52 µl) was added to the slides and incubated for 30 min at room temperature. The slides were washed in phosphate-buffered saline and counterstained with propidium iodide/antifade (supplied with kit) directly on the slide. Apoptotic cells were visualized by direct fluorescence detection of digoxigenin-labeled genomic DNA by epifluorescence microscopy. This method was based on the new 3'-OH DNA end generated by DNA fragmentation and typically localized in morphologically identifiable nuclei and apoptotic bodies. In contrast, normal nuclei, which had relatively insignificant numbers of DNA 3'-OH ends, were not stained with this reagent.

Preparation of cardiomyocytes. Cardiomyocytes were obtained by well-established methods (35). After experiments, hearts (control, ischemic, reperfused) were suculted and dissected with a 2-mm laser dissection microknife, quickly placed into a chilled dissociation buffer containing (in mM) 137 NaCl, 5.4 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 0.44 KH₂PO₄, 0.34 Na₂HPO₄, 5.6 dextrose, 20 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (pH 7.5), 50 U/ml penicillin, and 50 µg/ml streptomycin. The ventricles were cut into 1- to 2-mm cubes and dissociated by trypsinization (0.05% trypsin-EDTA at 37°C for 10 min). Unfused cells from the first treatment were discarded, and the sequence was repeated until all tissue was dissociated (~5 times). Freecells were collected in cold Dulbecco's modified Eagle's medium (GIBCO, Gaithersburg, MD) supplemented with 0.5% fetal calf serum and 0.002% deoxyribonuclease and washed in the same medium. The isolated primary cardiac myocytes were used for phospholipid topography, enzyme analysis, or flow cytometry.

DNA fragmentation. Apoptosis is best characterized biochemically by the cleavage of genomic DNA into nucleosomal fragments of 180 bp or multiples thereof that are readily detected as a DNA ladder by gel electrophoresis. DNA was isolated from cardiomyocyte (1 × 10⁶) to perform DNA laddering. Cardiomyocytes were pelleted in an eppendorf tube using 1,000 g for 2 min. The supernatant was aspirated. Twenty microliters of lysis buffer [10 mM EDTA, 0.5% sarsosyl, 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.0] were added, vortexed, and placed at 4°C for 15 min. One microliter proteinase K (stock solution 20 mg/ml) was added to each sample. The samples were vortexed and then incubated for 1 h at 40°C. After incubation for at least 1 h, 1 µl ribonuclease A (stock solution 10 mg/ml) was added and incubated for an additional hour at 37°C. Gel loading buffer (5 µl) was added to the sample. DNA samples were electrophoresed on a 1.8% agarose gel with ethidium bromide. DNA laddering was visualized and photographed under ultraviolet transillumination.

Assay of PE and PS topography in cardiomyocytes using 2,4,6-trinitrobenzenesulfonate (TNBS) has been extensively used to determine localization of PE and PS in the inner and outer leaflets of plasma membrane of intact cells. TNBS is a water-soluble nonfunctional chemical modifier that reacts with amino groups of phospholipids located on the outer surface of the cell membrane. Cardiomyocytes were incubated for 2 h at 4°C in a medium of the following composition (in mM): 120 NaHCO₃, 100 NaCl, and 2.5 TNBS (pH 8.2). The trinitrophenylation reaction was terminated by the addition of 40 mM Tris-HCl buffer, pH 7.4. The reaction mixture was then centrifuged at 1,000 g for 10 min, and the pellet was used for further extraction of lipids.

Extraction and high-performance thin-layer chromatography analysis of cell lipids. Total lipids were extracted from cardiomyocytes according to the Folch technique (9). Separation of both modified and nonmodified phospholipids was achieved through the use of two-dimensional high-performance thin-layer chromatography (HPTLC). Phospholipids from TNBS-labeled cells were separated on silica gel G plates (5 × 5 cm, Whatman) using two solvent systems: chloroform-methanol-28% ammonium hydroxide (65:35:5, vol/vol/vol) and chloroform-acetone-glacial acetic acid-water (50:20:10:5, by vol). Identification of phospholipids in the iodine-stained HPTLC plates was performed by comparison with authentic standards. In the TNBS-treated cells, unreacted phospholipids were detected by the ninhydrin reaction. The TNBS-reacted phospholipids were identified by their characteristic yellow color on HPTLC plates (13). These yellow spots were transferred to glass tubes and extracted two times with chloroform-methanol (2:1, vol/vol). Lipid phosphorus was determined according to Vaskovsky et al. (37). The content of TNBS-modified phospholipids was estimated as the ratio of the amount of modified phospholipid to the amount of total phospholipid (modified plus nonmodified) for each phospholipid class.

Statistical analysis. For statistical analysis, a two-way analysis of variance was performed with Scheffe's test was first carried out using Primer Computer Program (McGraw-Hill) to test for any differences between groups. If differences were established, the values were compared using Student's t-test for paired data. The values are expressed as means ± SE. The results were considered significant if P < 0.05.
ischemia

Corresponding propidium iodide (PI) staining is shown in red (Fig. 1, lane A, and B as well as in the ischemic hearts (Fig. 1, C and D). Even 30 min of ischemia (Fig. 1, E and F) could not induce apoptosis as evidenced under propidium iodide filter or under dual filter exposure (superimposing propidium iodide on fluorescence filter). Thirty minutes of ischemia causes irreversible cell injury and necrosis in rat heart. Surprisingly, there was no sign of tunnel of fragmented nuclear DNA in these biopsies.

Digoxigenin-labeled apoptotic cells were identified in the reperfused hearts. The extent of apoptosis increased with the progression of reperfusion time (Fig. 1, G–L). Apoptotic cell death was first evidenced after 30 min of reperfusion (Fig. 1, G and H). The number of apoptotic cells increased significantly after 90 min (Fig. 1, I and J) and 120 min of reperfusion (Fig. 1, K and L) as evidenced from the immunohistochemical staining of the extended DNA in these hearts.

In concert, as shown in Fig. 2, DNA fragmentation was very prominent at 120 min of reperfusion (lane G) compared with the control (lane B), ischemia (lanes C and D), and 30 min (lane E), 90 min (lane F), and 120 min of reperfusion (lane G). Ninety-minute reperfused hearts also showed some laddering, but it was not very prominent and did not appear in Fig. 2.

Membrane phospholipid patterns of cardiomyocytes obtained from the control hearts as well as from the hearts after ischemia-reperfusion are presented in Table 1. In cardiomyocytes, phosphatidylinositol and PE are the two abundant phospholipids representing ~45 and 37% of total phospholipids, respectively. Additionally, other phospholipids in the order of their abundance sphingomyelin > phosphatidylinositol > phosphatidylserine > diphosphatidylglycerol > lypo-phosphatidylcholine were detectable on the HPTLC plates. A significant increase in the content of lypo-phosphatidylcholine was observed in the cardiomyocytes after ischemia and reperfusion. There was no significant difference in distribution of other phospholipid classes between control cardiomyocytes and those after ischemia and/or ischemia-reperfusion.

A typical chromatogram of lipids from TNBS-treated cardiomyocytes is shown in Fig. 3. About 30% of total PE and 5% of total PS were available for TNBS in control cardiomyocytes (Figs. 3 and 4). The availability of both PE and PS was significantly increased (1.7- and 5-fold, respectively) in cardiomyocytes from the hearts after ischemia. There was no further increase in the availability of either PE or PS to TNBS in cardiomyocytes obtained from the hearts after ischemia-reperfusion (Fig. 4). This suggests that ischemia results in a significant loss of normal asymmetric sarcolemmal phospholipid distribution possibly with an outward migration of PE and PS.

Table 1. Phospholipid distribution of rat cardiomyocytes obtained from control, ischemic, and ischemic reperfused hearts

<table>
<thead>
<tr>
<th>Total Phospholipids, %</th>
<th>Control</th>
<th>Ischemia</th>
<th>Ischemia-reperfusion (30 min)</th>
<th>Ischemia-reperfusion (120 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipid Class</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>36.6 ± 2.4</td>
<td>36.2 ± 2.4</td>
<td>37.6 ± 2.4</td>
<td>36.9 ± 2.2</td>
</tr>
<tr>
<td>PS</td>
<td>3.8 ± 0.5</td>
<td>4.4 ± 0.5</td>
<td>4.1 ± 0.5</td>
<td>3.9 ± 0.5</td>
</tr>
<tr>
<td>PI</td>
<td>4.2 ± 0.5</td>
<td>4.3 ± 0.5</td>
<td>4.0 ± 0.5</td>
<td>4.1 ± 0.5</td>
</tr>
<tr>
<td>Sph</td>
<td>6.1 ± 0.5</td>
<td>6.3 ± 0.6</td>
<td>6.0 ± 0.5</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td>Dpg</td>
<td>3.6 ± 0.5</td>
<td>3.4 ± 0.5</td>
<td>3.2 ± 0.5</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>LPC</td>
<td>0.9 ± 0.3</td>
<td>1.8 ± 0.4</td>
<td>2.2 ± 0.4</td>
<td>2.1 ± 0.3</td>
</tr>
</tbody>
</table>

Values are mean percentage of total phospholipids ± SE (n = 6).

PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; Sph, sphingomyelin; Dpg, diphosphatidylglycerol; LPC, lysophosphatidylcholine.
DISCUSSION

In this study we have shown that reperfusion of ischemic myocardium is associated with apoptotic cell death in concert with DNA fragmentation. Additionally, our results have demonstrated for the first time that translocation of PE, in addition to PS, from the inner leaflet of the cardiomyocytes to the outer leaflet precedes apoptosis, suggesting that redistribution of PS and PE may serve as a signal for apoptotic cell death for myocardial reperfusion injury.

Numerous evidence exists that reperfusion of ischemic myocardium leads to a specific type of cellular injury generally known as reperfusion injury. Although the precise mechanism of reperfusion injury remains under considerable debate, three interrelated factors are believed to play a role in the pathophysiology of reperfusion injury. These are generation of oxygen-derived free radicals, intracellular Ca$^{2+}$ overloading, and loss of membrane phospholipids, all of which are associated with the reperfusion injury (3). Interestingly, the same three factors have been shown to be involved in the apoptotic cell death. The hallmarks of apoptosis are increased cytosolic Ca$^{2+}$ content (28), oxygen free radicals (14), and redistribution of membrane phospholipids (1, 8, 23). Taken together, these suggest an interesting possibility that reperfusion injury may be mediated by apoptosis.

A limited amount of evidence indicates that apoptosis can occur in cardiovascular tissues. For example, the results of a recent study demonstrated that hypoxia induces apoptosis in rat cardiomyocytes (33). Apoptosis has been shown to play a possible role in various cardiac disorders (16). In another related study, vascular smooth muscle cells were found to undergo apoptosis after infection with c-myc or adenovirus EIA gene (1). A recent study described immunohistochemical examination of the specimens retrieved from patients undergoing directional atherectomy for primary atherosclerotic lesions or recurrent arterial narrowing after percutaneous revascularization (15). The authors identified apoptotic cells in both types of vascular lesions. In the present study, we did not find any evidence for apoptosis after 30 min of ischemia when irreversible cellular injury begins to appear. Apoptotic cell death was visible after 90 min of reperfusion and became prominent after 2 h of reperfusion. Although our results implicate that reperfusion, and not ischemia, induces apoptosis, it is possible that ischemia initiated the signal for apoptosis that took a couple of hours to develop. This is supported by the fact that phospholipid redistribution occurred after 30 min of ischemia and did not change with reperfusion. It is also possible that both ischemia and reperfusion induce apoptosis, but it is not possible to distinguish these events in the present model.

The precise mechanism of PS translocation from the inner leaflet of the membrane to the outer leaflet is not fully understood. Several recent studies have demonstrated that apoptotic cells expose PS on the external leaflet of their plasma membrane (19). It is generally believed that such translocation is due to redistribution of existing phospholipids. Phospholipid topography (asymmetry) is maintained by a specific enzyme, aminophospholipid translocase, which is responsible for the translocation of PS (22). Because of the fact that this aminophospholipid translocation is an ATP-dependent process.
process (2) and that ATP is drastically reduced in the ischemic myocardium (30), a reduction of aminophospholipid translocase activity is likely to cause PS translocation to the outer leaflet. Moreover, this enzyme is known to be inhibited by Ca\(^{2+}\) (22). Numerous evidence exists to show intracellular Ca\(^{2+}\) overloading during ischemia and reperfusion (4). Thus increased intracellular Ca\(^{2+}\) in conjunction with reduced ATP level may decrease the aminophospholipid translocase activity of the myocytes, leading to the translocation of both PS and PE.

Nonrandom distribution of phospholipids over the two monolayers of plasma membrane is the characteristic of normal cardiomyocytes; a negatively charged PS is almost exclusively present in the cytoplasmic leaflet of the sarcolemma, which also contains the majority of PE (26). This asymmetric distribution is most likely caused by active transport of these lipids (by a Mg\(^{2+}\)-ATP-dependent protein with a high lipid selectivity, aminophospholipid translocase) and by interaction of the head group of these lipids with the cytoskeleton. Onset of ischemia initiates a sequence of events leading to a loss of normal asymmetric sarcolemmal phospholipid distribution with an outward migration of PE (24, 25, 27). In line with these observations, our results demonstrated that availability of PE to TNBS was significantly increased in cardiomyocytes from ischemic and reperfused hearts as compared with those from the controls.

Interestingly, even a much greater increase in the TNBS-labeled PS was detected in cardiomyocytes after ischemia and reperfusion; 25–30% of total PS appears to be available for the chemical modification. This, to the best of our knowledge, the first demonstration of the PE and PS externalization in plasma membranes of cardiomyocytes from ischemic and ischemic reperfused hearts. Studies from several laboratories demonstrated that early stage(s) of apoptosis may be associated with the loss of membrane phospholipid asymmetry and appearance of PS, normally found in the inner leaflet of plasma membrane (4, 26) on the outer surface of cells (18, 38, 39). The ability of apoptotic cells to trigger their own engulfment by phagocytic cells before cell lysis is crucial to the avoidance of the tissue damage and inflammation associated with necrosis (40). PS exposure on the outer cell surface is recognized by the macrophage scavenger receptors as an important signal for cell removal (8).

Although the apoptotic signaling function of externalized PS has been extensively characterized in different blood cells, its role in cardiomyocytes remains unknown. We speculate that externalized PS and PE is an early apoptotic signal in ischemic and reperfused hearts that is subsequently recognized by macrophages to facilitate the removal of damaged cardiac cells. Translocation of PE was never found for any other cell types. Externalization of PS and PE is observed in both ischemic and reperfused hearts, indicating that this event precedes DNA fragmentation and apoptosis, which occur only after prolonged reperfusion. Appearance of PS in the outer leaflet of plasma membrane was shown to occur significantly earlier than DNA laddering in lymphocytes and other blood cells (8, 39, 41). Finally, our results demonstrated simultaneous occurrence of nuclear fragmentation and apoptotic cell death in the ischemic reperfused myocardium. Apoptosis has been characterized biochemically on the basis of DNA fragmentation (40). However, evidence also exists to support the notion that apoptosis may occur in the absence of a nucleus. It is possible that reperfusion of ischemic myocardium causes nuclear damage and apoptotic cell death by independent pathways.

In summary, our results demonstrated apoptosis in concert with nuclear fragmentation in the ischemic reperfused myocardium. Both apoptotic cell death and DNA fragmentation occurred only after the reperfusion, whereas redistribution of cardiomyocyte phospholipids occurred during ischemia. Externalization of both PS and PE occurred after 20 min of ischemia and did not undergo further changes during reperfusion. For the first time, externalization of PE and PS to the outer leaflet is demonstrated in conjunction with reperfusion-induced apoptosis in the heart. This raises an interesting possibility that although apoptotic cells are visible during the reperfusion of ischemic myocardium, it may trigger the signal for apoptosis. Alternatively, membrane phospholipid redistribution may represent an effect of ischemic injury that is unrelated to apoptosis. However, available evidence as discussed above indicates that the former explanation is probably true.

An important question, however, is, What is the mechanism (the motive force) for such an externalization process? Several studies demonstrated that inhibition of aminophospholipid translocase and activation of ‘‘scramblase’’ are characteristic features of apoptosis (40, 41). Recently, using a newly developed procedure for specific probing of oxidative stress in membrane phospholipids in live cells (29), we found that peroxidation of PS precedes its externalization, DNA fragmentation, and apoptosis induced in murine leukemia 32D cells exposed to parquat (6). It is tempting to speculate that oxidation of PS and PE is functionally associated with their externalization in cardiomyocytes undergoing apoptosis. Indeed, numerous studies have demonstrated the development of oxidative stress and lipid peroxidation during the reperfusion of ischemic myocardium (5). Experiments are now underway to reveal specific oxidation of PS and PE in ischemic reperfused hearts.

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