Time Course of Changes in Porcine Myocardial Phospholipid Levels during Ischemia
A Reassessment of the Lysolipid Hypothesis

NISAR A. SHAikh AND EUGENE DOWNAR

SUMMARY This study was performed to determine the early and delayed metabolic effects of myocardial ischemia on the major membrane phospholipids and to reassess the potential role of lysophospholipids in the genesis of malignant dysrhythmias induced by ischemia. Samples taken from in situ hearts before and at various intervals up to 40 minutes after abrupt ligation of LAD were extracted by the classical Folch technique with modifications to avoid artificial lysophospholipid production and losses. Following thin layer chromatography of lipid extracts, phospholipid fractions were quantified by phosphorus estimation and lysophospholipids by a more sensitive method employing gas liquid chromatography. The total phospholipid content with the exception of lysophospholipids remained essentially constant throughout the early phases of acute ischemia, but fell by 6 and 14% after 8 and 24 hours, respectively. At 8 minutes, lysophospholipid levels in ischemic myocardium were significantly increased by 60% compared to pre-occlusion controls in the ischemic zone and by 25% in post-occlusion controls. They changed little thereafter. The molecular species of lysophospholipids remained unchanged throughout the period of ischemia studied. The mole fraction of other phospholipids as well as their fatty acyl and aldehyde profiles also were unchanged. Despite significant elevations in lysophospholipid levels, their absolute quantities were very small (0.6% of total phospholipid P) and 15-fold smaller than that reported in vitro to simulate electrophysiological manifestation of ischemia. However, such small amounts in vivo, if produced in the microenvironment of certain membrane-bound enzymes along with acidosis, hypoxia, and fatty acids, could be potentially deleterious to cell functions. Circ Res 48: 316-325, 1981

Many aspects of the metabolic changes associated with myocardial ischemia have been studied intensively in the past (see Opie 1969; Jennings, 1976). Despite early functional changes in membrane properties that are responsible for the well known electrophysiological manifestation of ischemia, little is known of myocardial phospholipid metabolism. The possibility that lysophospholipids contribute to ischemic damage in the heart was first suggested by Hajdu et al. (1957). Recently these lipids have been reported to accumulate in ischemic rabbit myocardium to an extent sufficient to produce electrophysiological changes similar to those seen in ischemia (Sobel et al., 1978; Corr et al., 1979). These studies led the authors to postulate a possible involvement of lysophospholipid in the genesis of malignant dysrhythmias induced by ischemia (Sobel et al., 1978). The pig, more than other animals, is finding increasing favor as a model for myocardial ischemia that is most relevant to the human heart. The immediate and delayed effects of abrupt coronary occlusion on the major phospholipid fractions, particularly the lysophospholipids, were therefore investigated in the pig. Preliminary results appeared elsewhere (Shaikh and Downar, 1980b).

The "lysolipid hypothesis" (Sobel and Corr, 1979) was reassessed by analyzing this lipid accurately and without artificial contaminations. Lysophospholipids can be artifactually produced if...
tissue containing acid-labile plasmalogenic phospholipids are extracted with acidified solvents (Shaikh and Downar, 1980a). In the present communication, lipids were extracted, along with internal standards, by a modification of the classical Folch technique (Folch et al., 1957) to circumvent the problems of artifactual intrapreparative lysophospholipid production, incomplete recoveries, and losses of certain other phospholipids. A highly reproducible assessment of lysophospholipid was then made by gas liquid chromatography.

**Methods**

All chemicals and solvents were reagent grade. Petroleum ether (b.p. 30-60°C) was refluxed over P2O5 and was triple distilled to obtain the 38-40°C fraction. Methanol was redistilled.

Chromatographically pure phospholipids were obtained from Serdary Research Laboratories. Cabbage phospholipase D, Naja Naja phospholipase A2, 1-heptadecanoyl-sn-glycerol-3-phosphorycholine (H-LPC) and 2,3-diheptadecanoyl-sn-glycerol-1-phosphorycholine were purchased from Sigma Chemical Co. Heptadecanoyl-glycerophosphorylcholine was purified from any phospholipid and free fatty acid contaminants by TLC using chloroform: methanol:acetic acid:water (C-M-AA-W, 100:45:20:6.8, by volume) as a developing solvent (Shaikh and Palmer, 1976). The lysophospholipid (lyso-PL) was isolated from the silica gel with repeated extraction with C-M-AA-W (50:39:1:10, by volume) and the extract washed with 1/3 volume of 4 M NH4OH (Arvidson, 1968), dried in vacuo, and quantitated by phosphate analysis. Gas-liquid chromatographic analysis showed heptadecanoic acid as the sole fatty acid constituent. Heptadecanoyl-glycerophosphorylcholine was prepared from diheptadecanoylglycerophosphorylcholine by transphosphatidyl migration using phospholipase D. Non-ischemic myocardium was sampled from a fixed site in the distribution of the obtuse branch of the circumflex artery, well outside the demarcation of the ischemic boundary. In these serial experiments, the non-ischemic control region was sampled before coronary artery occlusion ("pre-control") and again at the end of the ischemic period ("post-control"). In six pigs, no biopsy was taken until the end of the ischemic period when the whole left ventricle was rapidly excised and placed on ice. Two full thickness samples (10-15 g) were then taken with a circular biotome from the control (categorized as "post-control") and ischemic regions. The ischemic sample encompassed all the sites used for the serial-sample experiments. Over petri dishes containing dry-ice, approximately 2-mm-thick slices of endocardium, epicardium, and mid-ventricular wall were rapidly sliced by hand. All the samples were quickly rinsed in ice-cold saline, blotted dry, and frozen in liquid N2 before being weighed in the frozen state. The phospholipid composition data were expressed on a wet weight basis since it has been reported to give more constant values than the dry weight (Rouser et al., 1968).

**Lipid Extraction**

Lipids were extracted by the method of Folch et al. (1957) with some modification to eliminate phospholipid losses during purification of crude extracts (Kuklis, 1977; Nelson, 1969) and to achieve virtually complete recovery of all phospholipid fractions including lyso-PL (Shaikh and Downar, 1980a). This technique also circumvents any hydrolysis of vinyl ether-linked phospholipids (Plasmalogens) which results when tissues containing plasmalogens are extracted with acidified solvents. Approximately 100-150 mg of frozen myocardial tissue were placed in 6-ml ice-cold C-M mixture (2:1, vol/vol) containing 0.005% BHT (antioxidant) and homogenized in a Potter-Elvehjem homogenizer (kept in ice) as soon as the tissue began to soften. Appropriate amounts (0.75 ng P/mg tissue wt.) of H-LPC and H-LPE were added as internal standards. These standard lyso-PL were used to monitor the recoveries of myocardial lyso-PL from extraction.
mixture and subsequently to calculate the absolute amounts of corresponding lyso-PL after thin layer chromatography (TLC)-gas liquid chromatography (GLC). The homogenate was transferred to a 15-ml screw cap centrifuge tube. The homogenizer was rinsed twice with 3-ml portions of C-M mixture and the washes were added to the first homogenate to give a final 12-ml volume. After the homogenate had been shaken in Buchler Evapo-Mix for 15 minutes at room temperature, a biphasic mixture was produced by the addition of 3 ml of 0.9% aqueous NaCl solution. The tubes were shaken briefly and the two phases were produced by centrifugation. By blowing air gently, a Pasteur pipette was carefully inserted through the aqueous upper phase and interfacial protein residue and placed at the bottom of the tube. The lower lipid phase was then withdrawn without any contamination of the upper phase through this pipette by another Pasteur pipette and transferred to the round bottom flask. The remaining upper phase and protein residue were successively re-extracted three time each with 9-ml portions of "synthetic" lower phase (C-M:0.58% NaCl, 86:14:1 by volume). The lipid lower phase was removed after centrifugation as described above. A fourth and final extraction was made with synthetic lower phase containing 1 N HCl, the lower lipid phase was withdrawn and neutralized with ammonia vapor. All extracts were pooled in a round bottom flask and taken to near dryness in vacuo. The lipid residue was quantitatively transferred to a 7-ml glass vial using repeated extraction with C-M-W (75:25:2, by volume), dried under a stream of N₂, dissolved in 0.1 ml of moist chloroform, and subjected to TLC on the same day for lyso-PL isolation. The isolated lyso-PL were then quantified by their fatty acid content using GLC.

The recovery of phospholipids through use of the above extraction scheme was ascertained by extracting the myocardial tissue in the presence of ³²P-labeled rat liver phospholipid fractions of known radioactivity. For comparison, lipid extracts were also made with C-M (2:1, vol/vol) mixture and the extracts were not backwashed with aqueous salt solutions. Instead, the non-lipid contaminants were removed by partition chromatography over Sephadex G-25 columns (Nelson, 1972). Lipids were also extracted through use of acidified butanol by the method of Bjerve et al. (1974). After fractionation of lipid extracts by TLC, a measurement of the radioactivity of phospholipid fraction directly on silica gel scrapings was made by liquid scintillation spectrometry (Shaikh and Palmer, 1977).

**Thin Layer Chromatography of Phospholipids**

Since lyso-PL are relatively minor components of lipid extracts, several two-dimensional TLC per sample are required for one analysis. As a more practical alternative, lipid extracts were fractionated by a one-dimensional TLC technique. This system has several advantages, namely (1) a larger load of lipid can be applied in a shape of a band, (2) three samples can be fractionated per plate, and (3) complete fractionation of lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) from other major phospholipids can be achieved in a shorter time.

Lysophospholipids were fractionated on 0.3-mm thick layers of silica gel H plates, which were air dried and activated for an hour at 120°C before use. Lipid extract was quantitatively transferred and applied to TLC plates as an evenly spread 5-cm long band with several washings of the lipid containing vial with C-M-W (75:25:2, by volume). Pig liver LPC and LPE standards were also spotted as references for mobility of lyso-PL on TLC. Chromatograms were run at 22-23°C in a paper-lined tank containing C-M-AAA-W (75:60:10:8, by volume). Under these conditions, the sequence of phospholipid fractions on TLC were as follows: origin, LPC, sphingomyelin (Sph), choline phosphoglycerides (CPG), LPE, phosphatidylserine (PS), phosphatidylinositol (PI), ethanolamine phosphoglyceride (EPG), and cardiolipin (CL). PI and PS were often not well resolved, and CL fraction which moved along with the solvent front probably also contained phosphatidic acid (PA), phosphatidylglycerol (PG), and other neutral lipids. The purity of lyso-PL was checked by additional chromatographic techniques as described previously (Shaikh and Palmer, 1976). Phospholipid fractions were localized under UV after exposure to 0.05% 2',7'-dichlorofluorescein in M-W (3:1) mixture. Silica gel areas corresponding to lyso-PL were scraped and fatty acid methyl esters (FAME) were prepared on the silica gel scrapings by the alkali-catalyzed transesterification method of Glass (1971). To the scrapings in screw-capped tubes, 2 ml of 0.1 N sodium methoxide in dry methanol-benzene (6:4, vol/vol) containing 0.01% phenolphthalein were added and the tubes were mixed on a vortex mixer. After 30 minutes at room temperature, the tubes were transferred to ice and the reaction mixture was neutralized with 0.2 ml 1 N acetic acid in petroleum ether. Fatty acid methyl esters then were extracted with redistilled petroleum ether, washed with water, passed through anhydrous sodium sulfate mini-columns, and analyzed by GLC.

For quantitative purposes, phospholipids other than lyso-PL were fractionated by TLC on precoated silica gel HR plates (Anatech Inc.) as previously described (Shaikh and Palmer, 1976). Phospholipid fractions were localized on chromatograms by brief exposure to iodine vapor or with the acid-molybdate reagent of Dittmer and Lester (1964) and the phosphorus determined by the method of Bartlett (1959) directly on the lipid-containing silica gel (Shaikh and Palmer, 1976). Occasionally, phos-
pholipid composition was confirmed by two-dimensional TLC (Rouser et al., 1970) on 0.5-mm thick layers of silica gel H plates prepared with 3% aqueous Mg-acetate. When the fatty acid profile of individual phospholipids was required, the chromatogram was sprayed with dichlorofluorescein and the lipid-containing silica gel was refluxed with 6% sulfuric acid in methanol for 3 hours at 80°C for an acid-catalyzed transerstefication (Christie, 1972). The tubes were cooled on ice and the reaction mixture was brought to near neutrality with 4 M ammonium hydroxide. Fatty acid methyl esters and dimethyl acetals (DMA), corresponding to acyl and alk-1-enyl analogues, respectively (Farquhar, 1962), were extracted with petroleum ether as described above and analyzed by GLC.

Plasmalogen content was determined after isolation of CPG and EPG on silica gel H plates with 3% aqueous Mg-acetate. When the fatty acid profile of individual phospholipids was required, the chromatogram was sprayed with dichlorofluorescein and the lipid-containing silica gel was refluxed with 6% sulfuric acid in methanol for 3 hours at 80°C for an acid-catalyzed transesterification (Christie, 1972).

Gas Liquid Chromatography

Fatty acid methyl esters and DMA were analyzed by GLC (Kuksis, 1978) routinely performed on 6 ft. x 1/8 in. glass columns packed with 10% EGSS-x on 100-120 mesh Gas Chrom P (Applied Sciences Laboratories). The columns were installed in a F & M Scientific Model 402 Gas Chromatograph equipped with flame ionization detector. The carrier gas was nitrogen at 40 ml/min. Isothermal runs were made at 175°C with injector and detector heaters at 200 and 250°C, respectively. In several experiments, fatty acid profiles were confirmed by temperature programmed runs on 30-meter glass capillary columns coated with SP-1000 (Supelco) through use of Hewlett Packard model 5880A gas chromatograph equipped with a split mode injector, flame ionization detector, and Level 4 microprocessor/integrator. The runs were made in the range of 120-210°C at a heating rate of 5°C/min with zero grade helium as a carrier gas. The quantities of individual fatty acids (nmol) were calculated by comparing the peak areas with that of internal standard after appropriate corrections for the differences in the recovery of fatty acids from the column and in the sensitivity of the detector for different fatty acids were made.

Statistics

Significance of the results were determined by non-paired Student's t-test. P values of less than 0.05 were considered statistically significant.

Results

Recovery and Reproducibility of Phospholipid Extraction and Analysis

Since lyso-PL levels in various tissues are very low compared to levels of other phospholipids, it was essential to adopt an extraction scheme for cardiac tissue that would provide complete recoveries of lyso-PL without intrapreparative production of these compounds from plasmalogens. The extraction scheme adopted in this communication (modified Folch, see Methods) was tested for the recoveries of endogenous lyso-PL and of exogenously added 3P-labeled lyso-PL of known radioactivity. Table 1 compares the recoveries obtained with the modified Folch to that of other methods. For reasons discussed elsewhere (Nelson, 1969; Kuksis, 1977), the Folch extraction scheme using C-M mixture followed by a time-consuming purification on Sephadex G-25 column (impractical for a large number of analyses) was also used as a standard to evaluate other recoveries. The classical Folch method, which involves purification of crude lipid extracts in bipasic mixtures, resulted in 87-90% recovery of endogenous lyso-PL and 89-94% of exogenous radiolabeled lysocepholypoxidase. These recoveries could be improved to >98% if lipids were extracted by the modified Folch. In this scheme,

<table>
<thead>
<tr>
<th>Extraction scheme</th>
<th>No. of analyses</th>
<th>LPC nmol</th>
<th>LPC dpm</th>
<th>LPE nmol</th>
<th>LPE dpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folch/column</td>
<td>4</td>
<td>4.923 ± 0.052</td>
<td>7938 ± 48</td>
<td>7.401 ± 0.088</td>
<td>6831 ± 61</td>
</tr>
<tr>
<td>Classical Folch</td>
<td>4</td>
<td>4.341 ± 0.068</td>
<td>7063 ± 58</td>
<td>6.439 ± 0.102</td>
<td>6012 ± 72</td>
</tr>
<tr>
<td>Acid-butanol</td>
<td>6</td>
<td>4.798 ± 0.028</td>
<td>7921 ± 23</td>
<td>7.306 ± 0.040</td>
<td>6639 ± 43</td>
</tr>
<tr>
<td>Modified-Folch</td>
<td>6</td>
<td>79.17 ± 11.65</td>
<td>7941 ± 98</td>
<td>125.59 ± 20.10</td>
<td>6824 ± 110</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± se. Control myocardial tissue was homogenized in 0.9% NaCl at 4°C and equal portions of the homogenate representing 125 mg tissue wet weight were extracted under each protocol. Lyosphospholipids were then analyzed after TLC/GLC using internal standards (see Methods). Similar protocols were repeated with added 3P-labeled rat liver lyosphospholipids (6021 and 7004 dpm/kg P of LPC and LPE, respectively) and, after fractionation of phospholipids by TLC, radioactivity was determined directly on silica gel scrapings of appropriate fractions.
the first three successive neutral solvent extractions provided about 95% recovery of endogenous lysophospholipid and over 98% of exogenous labeled lysophospholipid. The final acidic extraction recovered virtually all of the remainder. The recoveries of total phospholipids, when compared to the Folch/column purification scheme, were better than 98% for six analyses. Similar recoveries (98.9 ± 1.7% for three determinations) were also obtained for exogenously added radiolabeled phospholipid fractions suggesting that, under the experimental conditions used, intraparative hydrolyses of phospholipids did not occur. The acid-butanol extraction scheme (Bjerve et al., 1974) resulted in a 12- to 19-fold increase in lysophospholipid levels even though the entire procedure was performed in a cold room on ice. The recoveries of added radiolabeled lysophospholipids remained unchanged. This suggested preferential hydrolysis of phospholipids containing vinyl ether linkages. Gas-liquid chromatographic analysis of fatty acyl profiles of lysophospholipids and their precursor phospholipid (CPE and EPG) confirmed such hydrolysis. A substantial decrease in DMA content of CPG and EPG was observed and was comparable to the increase in unsaturated fatty acids in lysocompounds (see Fig. 1).

Aliquots of normal myocardium were extracted in the presence of internal standards, and lysophospholipid were quantified by TLC/GLC. The reproducibility of the entire method of analysis is presented in Table 2. The maximum variation from the mean was less than 1% for lysophospholipid and 3% for total phospholipids, and the difference between highest and lowest levels was less than 1.5% and 5%, respectively. This highly reproducible method for the quantitation of lysophospholipid was adopted for all subsequent analyses.

Lysophospholipid Levels of Normal and Ischemic Myocardium

Lysophospholipid levels in normal and ischemic myocardium are presented in Table 3. Prior to occlusion, levels of both LPC and LPE were very low (0.15% of total phospholipids) and did not vary much (<3%) among different hearts. Following 8 minutes of coronary occlusion, the levels increased by about 60% compared to their own pre-control values or the mean of the pre-controls. In a few instances,
endocardium was isolated for analysis. The lyso-PL levels in these cases did not appear to differ from those observed in transmural samples.

In normal myocardium, the molecular species of LPC is made up of 70% saturated and 30% unsaturated entities, whereas in LPE, the distribution is about 50% each (Table 4). In the ischemic myocardium where the total content of lyso-PL increased, the distribution of the individual molecular species did not alter. This suggests that the increases in lyso-PL levels were not restricted to certain molecular species but were evenly spread among all molecular entities.

Total phospholipid content of myocardium remained essentially constant throughout the first 40 minutes of ischemia (P > 0.50). In two pigs in which coronary occlusion was maintained for 8 and 20 hours, the total phospholipid content/g tissue wet weight fell by 6 and 14%, respectively. The molar proportions of individual phospholipid fractions did not alter at any time with the exception of lysocom-

### Table 2 Reproducibility of Phospholipid Extraction and Analyses

<table>
<thead>
<tr>
<th>Analysis no.</th>
<th>LPC (nmol)</th>
<th>LPE (nmol)</th>
<th>Total phospholipid (μg P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.822</td>
<td>7.379</td>
<td>102.1</td>
</tr>
<tr>
<td>2</td>
<td>4.755</td>
<td>7.315</td>
<td>98.6</td>
</tr>
<tr>
<td>3</td>
<td>4.626</td>
<td>7.419</td>
<td>103.3</td>
</tr>
<tr>
<td>4</td>
<td>4.789</td>
<td>7.321</td>
<td>101.7</td>
</tr>
<tr>
<td>5</td>
<td>4.613</td>
<td>7.371</td>
<td>101.0</td>
</tr>
<tr>
<td>6</td>
<td>4.780</td>
<td>7.323</td>
<td>102.8</td>
</tr>
<tr>
<td>Mean</td>
<td>4.788</td>
<td>7.356</td>
<td>101.6</td>
</tr>
<tr>
<td>±SD</td>
<td>0.029</td>
<td>0.040</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SD of (n) analyses. Aliquots of myocardial homogenate (see legend Table 1) representing 125 mg tissue wet weight were extracted along with internal standards by the modified-Folch technique. After fractionation of lipid extracts by TLC, lyso-PL were analyzed by GLC and other phospholipid fractions were quantified by phosphate analysis.

#### Table 3 Lyso phospholipid Content of Porcine Myocardium

<table>
<thead>
<tr>
<th>Time ligation (min)</th>
<th>Normal zone</th>
<th>Ischemic zone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LPC (nmol/g wet wt.)</td>
<td>% change</td>
</tr>
<tr>
<td>0 (Pre-control)</td>
<td>40.3 ± 1.3 (22)</td>
<td>17.6</td>
</tr>
<tr>
<td>2-40 (Post-control)</td>
<td>51.0 ± 2.6 (19)</td>
<td>26.6</td>
</tr>
<tr>
<td>2</td>
<td>47.4 ± 2.9 (9)</td>
<td>17.6</td>
</tr>
<tr>
<td>4</td>
<td>49.0 ± 3.9 (6)</td>
<td>21.6</td>
</tr>
<tr>
<td>6</td>
<td>62.5 ± 2.2 (10)*</td>
<td>57.6</td>
</tr>
<tr>
<td>12</td>
<td>50.6 ± 5.2 (11)</td>
<td>25.6</td>
</tr>
<tr>
<td>20</td>
<td>55.8 ± 3.6 (7)*</td>
<td>38.5</td>
</tr>
<tr>
<td>40</td>
<td>55.2 ± 2.6 (8)*</td>
<td>37.0</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SD of (n) analyses. All values are significantly different (P < 0.001) than respective pre-controls (LPC and LPE) and post-control (LPE).

*Significantly different from LPC post-control (P < 0.05).

**Discussion**

In vivo concentrations of lyso-PL in porcine myocardium are very low. Artifactual lyso-PL can be produced readily if tissue storage is prolonged even at -20°C (Rouser et al., 1968) and by careless
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Table 5  Phospholipid Composition of Normal and Ischemic Myocardium

<table>
<thead>
<tr>
<th>Time ligation</th>
<th>Normal zone</th>
<th>Ischemic zone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(0 min)</td>
<td>(20-40 min)</td>
</tr>
<tr>
<td>Total phospholipids</td>
<td>895.3 ± 43.1</td>
<td>813.6 ± 52.6</td>
</tr>
</tbody>
</table>

Phospholipid fractions* (%) of total lipid-P

<table>
<thead>
<tr>
<th>Component</th>
<th>Normal</th>
<th>Ischemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPG</td>
<td>37.35</td>
<td>37.10</td>
</tr>
<tr>
<td>Dipalmitoyl-PA</td>
<td>38.16</td>
<td>37.22</td>
</tr>
<tr>
<td>Plasmalogen</td>
<td>25.34</td>
<td>25.68</td>
</tr>
<tr>
<td>Dipalmitoyl-PS</td>
<td>12.16</td>
<td>12.26</td>
</tr>
<tr>
<td>Plasmalogen</td>
<td>13.18</td>
<td>13.42</td>
</tr>
<tr>
<td>Choline phosphoglycerides (CPG)</td>
<td>19.24</td>
<td>18.73</td>
</tr>
<tr>
<td>Ethanolamine phosphoglycerides (EPC)</td>
<td>6.29</td>
<td>7.12</td>
</tr>
<tr>
<td>Phosphatidylserine (PS)</td>
<td>4.34</td>
<td>4.12</td>
</tr>
<tr>
<td>Cardiolipin (CL)</td>
<td>6.04</td>
<td>6.18</td>
</tr>
<tr>
<td>Phosphatidylinositol (PI)</td>
<td>0.18</td>
<td>0.19</td>
</tr>
<tr>
<td>Sphingomyelin (Sph)</td>
<td>0.38</td>
<td>&lt;0.60</td>
</tr>
<tr>
<td>PE</td>
<td>0.15</td>
<td>0.19</td>
</tr>
<tr>
<td>LPC</td>
<td>0.23</td>
<td>0.28</td>
</tr>
<tr>
<td>LPE</td>
<td>0.23</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Abbreviations used: CPG, choline phosphoglycerides; EPC, ethanolamine phosphoglycerides; CL, cardiolipin; PI, phosphatidylinositol; PS, phosphatidylserine; Sph, sphingomyelin; PA, phosphatidic acid; PG, phosphatidylglycerol. Total phospholipid content was calculated by adding phosphate content of individual phospholipid fractions with that of LPC and LPE amounts which were estimated by TLC/GC as described in Methods.

* Mean of duplicate analyses of three independent samples. Data of the ischemic zone did not differ from that of normal zone (P > 0.50) with the exception of 24-hour samples which represents the mean of three independent analyses of the same pig.

Table 6  Composition of Acyl and Alkenyl Moieties from Porcine Myocardial Phospholipids of Normal and Ischemic Zones*

<table>
<thead>
<tr>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPG</td>
</tr>
<tr>
<td>EPG</td>
</tr>
<tr>
<td>CL</td>
</tr>
<tr>
<td>PI</td>
</tr>
<tr>
<td>PS</td>
</tr>
<tr>
<td>Sph</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component</th>
<th>Normal</th>
<th>Ischemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>12.68</td>
<td>13.48</td>
</tr>
<tr>
<td>16:0</td>
<td>18.37</td>
<td>3.15</td>
</tr>
<tr>
<td>18:0</td>
<td>4.36</td>
<td>4.36</td>
</tr>
<tr>
<td>18:1</td>
<td>18.73</td>
<td>18.73</td>
</tr>
<tr>
<td>16:1</td>
<td>0.90</td>
<td>0.84</td>
</tr>
<tr>
<td>18:0</td>
<td>1.05</td>
<td>0.61</td>
</tr>
<tr>
<td>18:1</td>
<td>1.37</td>
<td>1.58</td>
</tr>
<tr>
<td>20:0</td>
<td>6.80</td>
<td>6.66</td>
</tr>
<tr>
<td>22:0</td>
<td>1.98</td>
<td>1.93</td>
</tr>
<tr>
<td>24:0</td>
<td>2.73</td>
<td>2.76</td>
</tr>
<tr>
<td>26:0</td>
<td>0.74</td>
<td>0.96</td>
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</tbody>
</table>

* Samples were taken from normal and ischemic zones after 40 minutes of LAD occlusion and are mean of three analyses: DMA, dimethylacetals representing alkenyl moieties; tr, trace; Longer chain fatty acid, e.g., 20:3, 20:5, 22:5 in CPG, EPG, PI, and PS, were less than 1% and are not recorded.

† Carbon length = number of unsaturated bonds.
phospholipids (e.g., lyso-PL, PI, PS, CL, etc.). The use of alkaline solvents (Rouser et al., 1963) also produces decacylated products in small amounts. Phospholipids, mainly acidic, can also be lost during biphasic purification of lipid extracts (Table 1; Kuk-sis, 1977).

In the present study, myocardial samples were quickly frozen in liquid nitrogen and then immediately subjected to lipid extraction without further delay to avoid postmortem and intrapreparative hydrolysis of phospholipids. Incomplete recoveries and plasmalogenic hydrolysis were avoided by a suitable adaptation of the classical Folch lipid extraction technique. Losses due to physical handling of the lipid extracts (e.g., extraction from round bottom flasks, transfer to TLC plates, transmethylation process, and extraction of FAME, etc.) were corrected for by the use of appropriate internal standards (see Methods). Consequently, a high degree of accuracy and reproducibility was achieved in the analyses of total phospholipids and lyso-PL in porcine myocardial tissue (Tables 2 and 3).

The total phospholipid content of porcine myocardium is in close agreement with previous reports on ungulates (see White, 1973). However, LPC levels of normal porcine myocardium reported in this study are lower than those reported for different organs and heart muscle of other mammalian species (0.15% vs. 0.4–1.6% of total lipid-P). LPE content was not detectable in the heart muscle of other mammals and often not analyzed in other organs. Levels of LPC and LPE in the human heart (1 hour 45 minutes postmortem) were 3.5 and 1.5% of total lipid-P, respectively (Simon and Rouser, 1969), which is higher than reported here for porcine myocardium. All these studies were not directed principally toward the analyses of lyso-PL, and their higher values might be attributable to the intrapreparative/postmortem production of such compounds. There has been no specific assessment of lyso-PL in any myocardial tissue other than rabbit myocardium (Sobel et al. 1978). That study showed levels of lyso-PL manifolds (20–35×) greater than those presented here for pig myocardium. This difference may, in part, be due to species variation but is more likely due primarily to the methodology used. The rabbit myocardium was extracted with acidified butanol over a period of 2 hours. This would be expected to result in the hydrolysis of acid-labile plasmalogens, thereby producing lysocompounds. The authors, however, attempted to refute this possibility with a strong argument based on the fatty acid profiles of lyso-PL, their structural analyses, and the chromatographic behavior of monoacylglycerols enzymatically produced in vitro from lyso-PL. Porcine myocardium, when extracted with acidified butanol for 10 minutes, produced an artifactual increment of 12- to 20-fold in lyso-PL levels that were about one-half of that reported for rabbit myocardium. These lyso-PL, upon fatty acid analysis, revealed a very high content of unsaturated fatty acids which was consistent with hydrolysis of plasmalogens (see Fig. 1). Furthermore, analyses of the precursor phospholipids (CPC, EPO) showed a reciprocal decrease in the aldehyde content, confirming that vinyl ether linkages had been hydrolyzed with acidified butanol. Thus, there is a strong possibility that the much higher levels of lyso-PL reported in rabbit myocardium were of intrapreparative origin.

Myocardial ischemia produces certain well known metabolic effects such as accumulation of K+, lactate, AMP, ionsosine, and inorganic phosphate (see Opie, 1969; Jennings, 1976). Recently, Sobel et al. (1978) reported that lyso-PL levels in rabbit myocardium also increased during ischemia. In porcine myocardium, a 60–70% increase in lyso-PL levels occurred but, as already stated, the concentrations were very much lower. Lyso-PL concentrations in tissues are determined by the interplay of phospholipid-metabolizing enzymes. Membrane-bound phospholipases can produce lyso-PL which, in turn, can either be re-acylated or transacylated, depending upon the energy state of the cell, to form precursor PL or further degraded (Van den Bosch, 1974). Normally their concentration is maintained very low, since lyso-PL in sufficient quantities are potent detergents and could alter general properties of the membrane such as fluidity and permeability (see Weltzein, 1979). In in vivo studies, they have been shown to be vasopressors (Takumura et al., 1978) and have also been implicated in the genesis of malignant arrhythmias (Corr et al., 1979). The lyso-PL levels seen in ischemic porcine myocardium represent <0.6% of total phospholipids and are much lower than those shown to have deleterious electrophysiological effects. Such concentrations, if distributed evenly over the entire tissue membranes, are not likely to produce changes in membrane permeability. On the other hand, if these accumulations are restricted to certain parts of the membrane or subcellular organelles, they could be sufficient to modify the microenvironment of certain enzymes so as to produce deleterious effects on cell function. Lysophospholipids have been shown to affect directly the activities of enzymes such as sarcolemmal Na+-K+-ATPase and galactosyltransferase (Moorkerjea and Yung, 1974; Shier and Trotter, 1976; Owens et al., 1979). A number of other enzymes have been shown to require phospholipids for their activities (see Finean, 1973).

The small but significant elevation of lyso-PL in non-ischemic porcine myocardium is surprising. It may reflect a general activation of myocardial phospholipase activities due to indirect effects of ischemia, such as an increase in cardiac sympathetic tone (Malliani et al., 1969). Although direct stimulation of phospholipases has yet to be described, catecholamines produce an increase in intracellular Ca2+ levels, and phospholipase activation has been shown to be Ca2+-dependent. In ischemic myocardium, in addition to elevated Ca2+, other factors...
such as hypoxia, low levels of high energy phosphates, and bradykinin release may also lead to activation of phospholipases. These factors, together with the lack of washout, result in a greater accumulation of lyso-PL. The fact that lyso-PL levels (LPC + LPE) did not continue to increase beyond 8 minutes of ischemia suggests that, after reaching a critical concentration, they are degraded further by lysophospholipases and other enzymes.

The molecular species of lyso-PL (LPC, LPE) in ischemic myocardium did not differ from that of normal myocardium. The effect of ischemia therefore is probably mediated by an activation of phospholipases that is not species specific. It also indicates that the elevation in lyso-PL levels was not due to entrapment of platelets and erythrocytes, since these would be expected to alter the fatty acids profiles.

Ischemia of up to 40 minutes in duration did not produce any change either in total phospholipid content or in the individual phospholipid fractions. When these were analyzed for fatty acyl and aldehyde content, no significant change was observed. This suggests that, in general, the metabolic integrity of the membranes was maintained. The decreases in the total phospholipid content seen after 8 and 24 hours of ischemia may reflect the change in tissue weight due to edema and/or a greater rate of membrane phospholipid degradation, probably due to leaky lysosomes (see Hoffstein et al., 1976).

Substantial decreases in some phospholipid fractions of subcellular organelles obtained from ischemic rat liver and canine myocardium favor the latter view (Chien et al., 1978; Vasdev et al., 1979). The observation that the mole fraction of individual phospholipids of 24-hour ischemic myocardium did not appear to alter indicates that the phospholipid degradation was not selective.

In conclusion, although early ischemia produces a significant elevation of lyso-PL in porcine myocardium, the absolute quantities are very small and alone cannot be responsible for the electrophysiological changes of ischemia. The functional significance of these elevations in the presence of other concomitant changes associated with ischemia remains to be determined.

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