Protection by Verapamil of Mitochondrial Glutathione Equilibrium and Phospholipid Changes During Reperfusion of Ischemic Canine Myocardium

Kiminori Kajiyama, Daniel F. Pauly, Helen Hughes, Seung Boo Yoon, Mark L. Entman, and Jeanie B. McMillin-Wood

Pretreatment of the ischemic myocardium with verapamil protects against mitochondrial respiratory depression observed during ischemic arrest as well as during reperfusion. Since ischemic mitochondrial function appears not to be altered further by reperfusion, the purpose of this study is to identify a biochemical event affecting mitochondria that is specifically associated with reperfusion injury. It has been proposed that increased cellular Ca\(^{2+}\) influx and oxygen toxicity may result from reintroduction of coronary flow. Increased cytosolic Ca\(^{2+}\) is transmitted to the mitochondria with subsequent activation of Ca\(^{2+}\)-dependent events, including phospholipase A\(_2\). Net production of lyso phospholipids (and loss of total diacylphospholipids from the mitochondria) will proceed when reacetyl mechanisms are inhibited. Since acyl-CoA:lyso phospholipid acyltransferase is a sulfhydryl-sensitive enzyme and since increased activity of glutathione peroxidase shifts the levels of the mitochondrial sulfhydryl buffer, glutathione, towards oxidation, levels of glutathione and its oxidation state were measured during reperfusion in the absence or presence of verapamil pretreatment. Ischemia lowers total glutathione and reduces the redox ratio (reduced glutathione: oxidized glutathione) by 85%. Reperfusion partially returns the redox ratio to control by causing oxidized glutathione to disappear from the matrix. Verapamil maintains both the concentration and the redox potential of glutathione at control levels. Concomitant with alterations in reduced glutathione: oxidized glutathione is a decrease in ischemic mitochondrial phospholipid content. During reperfusion, phosphatidylethanolamine and its major constituent fatty acids (C\(_{18}:0\) and C\(_{20}:4\)) are specifically lost from the mitochondrial membrane. Accompanying the significant loss of arachidonic acid during reperfusion is the decreased content of 11-OH, 12-OH, and 15-OH arachidate. These lipid peroxidation products are not increased in ischemia. It is proposed that oxidation of matrix glutathione to glutathione disulfide during ischemia results in formation of glutathione-protein mixed disulfides and inhibition of sulfhydryl-sensitive proteins, including acyl-CoA lysophosphatidyle acetyltransferase. Thus, metabolic events occurring within the ischemic period set the stage for prolonged dysfunction during reperfusion. (Circulation Research 1987;61:301–310)

Ischemic damage to the heart is associated with changes in mitochondrial ultrastructure in situ and in oxidative function of isolated mitochondria in vitro. Early changes present during periods of reversible damage include mitochondrial swelling and matrix clearing. As myocardial injury proceeds to the stage of irreversibility, mitochondrial membrane rupture and fragmentation of the cristae occur.\(^1\) Reperfusion of irreversibly injured myocardium results in cellular swelling and further increase in the number of granular densities present in the mitochondria.\(^2\) The dense bodies that accumulate during the reperfusion interval are believed to result from increased Ca\(^{2+}\) influx into the cell and subsequent Ca\(^{2+}\) sequestration by the mitochondria.\(^3,4\) This interpretation is given further support by the protective action of calcium channel blocking agents, e.g., verapamil, in reduction of ultrastructural damage to the cell as well as in preservation of mitochondrial structure\(^5,6\) and function.\(^7\) The observed changes in ischemic myocyte permeability to Ca\(^{2+}\) may be related to degradation and depletion of sarcemmal membrane phospholipids.\(^8\) Elevated concentrations of total tissue linoleic and arachidonic acids in ischemic heart may reflect activation of membrane phospholipase activities.\(^9,10\) Although there is a correlation between the appearance of nonesterified fatty acids in the myocyte and the onset of cell membrane alterations, Van der Vusse et al\(^11\) note that the cellular membrane responsible for the release of arachidonate has not been defined. An alternate mechanism that may lead to sarcoemmal membrane...
disruption and explosive cell swelling during reperfusion of the irreversibly ischemic myocardium is the generation of oxygen free radicals that may accompany the reinduction of coronary flow to the heart. In this case, membrane degradation may result from stimulated phospholipase activity due to formation of lipid peroxides, with consequent excision of the affected polyunsaturated fatty acids. Recent data also suggest that oxygen free radicals may be partially involved in the reversible ischemic injury that occurs during short periods of coronary occlusion followed by reperfusion.

Despite the pronounced effects of reperfusion on cellular structure in the irreversibly injured myocardium, the event leading to the morphologic expression of increased damage during reperfusion probably occurs before reinduction of coronary flow. Studies on isolated mitochondria have provided little or no evidence for acceleration of mitochondrial dysfunction as a result of reperfusion. Furthermore, pretreatment of the myocardium with verapamil or diltiazem preserves mitochondrial ultrastructure and isolated mitochondrial function in vitro during both ischemia and reperfusion. These results suggest that protection of the myocardium during the ischemic interval is necessary to prevent expression of cell damage during reflow. A role for mitochondria in the genesis of the irreversible state was originally proposed by Jennings. This suggestion was strengthened by documentation of functional abnormalities in mitochondria isolated after brief periods of ischemia, i.e., 5-15 minutes following coronary occlusion. Attempts to establish a relation between respiratory activity in isolated mitochondria and ischemic injury have been criticized because loss of mitochondrial protein occurs during isolation from severely ischemic tissue. It may be argued that the isolation procedure "selects" for a more normal population of mitochondria. Nonetheless, observations are consistent in that the degree of respiratory impairment of isolated mitochondria parallels the severity of contractile dysfunction and that reversal of the ischemic depression in physiologic performance by calcium antagonists is accompanied by a reversal in mitochondrial phosphorylating respiration. However, none of the mitochondrial functional indexes measured thus far have been directed at the possible manifestations of reperfusion injury that may be distinguishable from perturbations due to ischemia alone.

Turnover of mitochondrial phospholipids represents one potential area of investigation in which alterations in membrane lipid composition are known to arise from a combination of oxidative stress and increased cellular Ca²⁺ via the Ca²⁺-dependent phospholipase A₂ present in the mitochondrial inner membrane. During the detoxification of oxygen free radicals, increased production of oxidized glutathione (GSSG) may result from enhanced oxidation of reduced glutathione (GSH) by glutathione peroxidase. Alternatively, GSSG conversion to GSH may be decreased to the mitochondrial dehydrogenases and transhydrogenase by a limiting supply of reducing equivalents. Subsequent changes in mitochondrial thiol: disulfide ratios may have direct effects on sulfhydryl-dependent processes. In the specific case of phospholipid turnover, the enzyme acyl-CoA lysophospholipid acyltransferase is thought to be particularly sensitive to alterations in the matrix GSH: GSSG ratio. Thus, in the face of cellular oxidative stress and Ca²⁺ influx during reperfusion of the ischemic heart, the products of phospholipid breakdown should accumulate or increase over that observed during ischemia alone. In this investigation, we report specific changes in mitochondrial phospholipid and fatty acid composition due to 60 minutes of ischemia alone or ischemia followed by reperfusion. Changes in mitochondrial glutathione redox status, as an index of oxidative stress, and levels of lipid peroxidation products are also quantitated as possible indicators of peroxidative events. Finally, since verapamil protection may be linked to the preservation of cellular Ca²⁺ homeostasis, the effect of this agent on phospholipid turnover in the ischemic/reperfused myocardium also is reported.

Materials and Methods

Experimental Model and Protocol

A total of 24 dogs (15-20 kg) of either sex were studied. Ischemia was produced as previously described by this laboratory. Following sodium pentobarbital anesthesia (30 mg/kg), the dogs were intubated and ventilated with a Harvard respiratory pump to maintain O₂ and CO₂ partial pressures within normal physiologic limits. A left thoracotomy via the fifth intercostal space allowed exposure of the circumflex (CFX) coronary artery for placement of a Doppler flow probe and a "reversible" ligature. The electrocardiograph was monitored with a standard surface electrocardiograph.

Four groups of dogs were studied as follows: Group 1, control: hearts were removed following anesthesia (7 dogs); Group 2, ischemic: hearts were removed following 60 minutes of total CFX occlusion (6 dogs); Group 3, ischemic and reperfused: hearts were removed following 60 minutes of total CFX occlusion with subsequent blood reperfusion for 20 minutes (6 dogs); Group 4, ischemic and reperfused following verapamil pretreatment: verapamil (0.3 mg/kg) was infused 15 minutes prior to occlusion and hearts were removed following 60 minutes of ischemia, followed by 20 minutes of blood perfusion (5 dogs).

Mitochondrial Preparation and Assay

Mitochondria were prepared from 15-20 g of control, ischemic, and ischemic/reperfused (± verapamil) left ventricle according to the procedure of Palmer et al as modified in this laboratory for dog cardiac muscle. This procedure releases two fractions of mitochondria from the heart following either polytron homogenization or treatment with the protease nagarse. By this differential isolation procedure, the mitochondria initially isolated by gentle polytron homogenization are more sensitive to ischemia as reflected by decreased rates of phosphorylating respiration and by
an inability to retain small quantities of calcium added to the incubation medium.\textsuperscript{25} Both mitochondrial pre-
parations were washed twice and suspended in a medium
containing (in mM) mannitol 220, sucrose 70, MOPS
5, pH 7.4 at a protein concentration of 30–40 mg/ml.
Protein was determined by the biuret method.\textsuperscript{26}

\textbf{Mitochondrial Respiration and Ca\textsuperscript{2+} Flux}

\textit{Measurements}

Mitochondrial respiration was measured at 30\degree C with a Model 53 oxygen monitor, Yellow Springs Instruments, Yellow Springs, Ohio. The respiratory
medium contained sucrose 0.25 M, Tris (hydroxy-
methyl) aminomethane 10 mM, and potassium phos-
phate 12.5 mM, pH 7.4, bovine serum albumin
(fraction V) 0.1\%, and ethyleneglycol-bis (aminoethyl-
ether)-N,N\textprime- tetraacetic acid (EGTA) 1 mM in a total
volume of 2 ml. Respiratory substrates were present at the final concentrations and combinations indicated (in mM): glutamate 7.5, malate 3.75, and succinate 7.5
(plus 5 \mu g rotenone/mg protein). Mitochondrial protein
was present at a final concentration of 1 mg/ml, and
300–450 nmol of adenosine diphosphate (ADP) was
added to initiate phosphorylating respiration (state 3
QO).

Mitochondrial Ca\textsuperscript{2+} uptake and release was assayed
as previously described by our laboratory\textsuperscript{27} using an
Amino DC-2 spectrophotometer (Silver Springs,
Md.) in the dual beam mode. Antipyrylazo III (K
and K Laboratories, Plainview, N. Y.) was present at a final
concentration of 50 \mu M, and Ca\textsuperscript{2+} movements were
monitored at the wavelength pair 620–642 nm. Ca\textsuperscript{2+}
uptake and release by mitochondria (1 mg) was
initiated by the addition of 10 \mu M Ca\textsuperscript{2+} followed by either 3 \mu M ruthenium red alone to inhibit energy-
dependent Ca\textsuperscript{2+} uptake (ruthenium-red-insensitive
Ca\textsuperscript{2+} release) or by 3 \mu M ruthenium red plus 10 mM
Na\textsuper{+} to initiate Na\textsuperscript{+}–Ca\textsuperscript{2+} exchange. The rates
of ruthenium-red-insensitive Ca\textsuperscript{2+} release were recorded
before Na\textsuperscript{+} addition. The increment in Ca\textsuperscript{2+} efflux
observed after injection of Na\textsuperscript{+} represents Na\textsuperscript{+}–
dependent Ca\textsuperscript{2+} release.\textsuperscript{28}

\textbf{Mitochondrial Lipid Content}

The remainder of the mitochondrial suspension was used to assay for phospholipids and total fatty acids.
Phospholipids were extracted from mitochondrial sus-
pensions in the presence of 10 \mu M EGTA according to
the method of Folch et al.\textsuperscript{29} Mitochondrial suspensions
were extracted twice with 20 volumes of chloro-
form: methanol (2:1, vol/vol). The organic extracts were
taken to dryness under a stream of nitrogen and the
residues dissolved in chloroform: methanol (2:1,
vol/vol). Aliquots of the samples were applied to
silica gel type-60 thin layer chromatography plates and
developed in chloroform: methanol: acetic acid:
water (55:35:3:2, vol/vol) in the first dimension and
chloroform: acetone: methanol: acetic acid: water
(45:16:15:11:6, vol/vol) in the second dimension.\textsuperscript{30,31}
The phospholipids were visualized by exposure to iodine
vapor and identified by comparison with the migration of phospholipid standards. The identified spots were scraped
off and digested with 70% perchloric acid (0.5 ml) for 1
hour at 180\degree C. After cooling, 1 ml of water was added
to each sample, and lipid phosphorus content was
determined according to Rouser et al.\textsuperscript{30}

For determination of total fatty acid content of the
mitochondrial samples, 0.2 \mu mol heneicosanoic acid
(C21:0) was added as an internal standard prior to the
Folch extraction procedure. Assays were performed in
duplicate. The dried residues from the Folch extracts
were mixed with 1 ml of methanolic base, heated in a
steam bath for 3 minutes, mixed with 1 ml of boron
trifluoride, heated for a further 3 minutes, and extracted
with petroleum ether.\textsuperscript{31} The petroleum ether was taken
to dryness and the residue dissolved in carbon disulfide
and run on a Hewlett-Packard 5830A gas chromatog-
raph at 285\degree C using a sililar column (Supelco,
Bellefonte, Penn.). Retention times were determined
and the percentage of material in each peak was
calculated. To convert percentage to moles of fatty
acids, peaks were compared with the internal standard.

Lipid peroxidation products were quantitated ac-
cording to the procedure of Hughes et al.\textsuperscript{32} Lipid
extracts were prepared as described,\textsuperscript{27} and any
hydroperoxides present were reduced to the more stable
hydroxy derivatives by addition of triphenyl-
phosphine. Methyl-15-hydroxyarachidate (0.3
nmol) was added as an internal standard. Following
transmethylation, the hydroxy fatty acid methyl
esters were isolated by silicic acid chromatography and
derivatized to their trimethylsilyl ethers prior to
analysis by gas chromatography–mass spectrometry.
Three isomers, 11-, 12- and 15-hydroxyeicosatetra-
enoic acid were quantitated using selected ion
monitoring techniques relative to the internal stan-
dard, methyl 15-hydroxyarachadate.

\textbf{Quantitation of Glutathione and Glutathione Disulfide}

Reduced and oxidized glutathione were resolved by
anion exchange and high performance liquid chroma-
tography and were detected by an in-line, recycling
post column reaction.\textsuperscript{33} Following perchloric acid
precipitation of mitochondria and removal of KC1O
from the neutralized extracts, GSH and GSSG were
separated by high performance liquid chromatography
on a Toyosota TSK-DEAE SPW (Cel Associates,
Houston, Tex.) anion exchange column. The effluent
was analyzed by a recycling assay (sensitivity in-
creased 20-fold) that is sensitive to substrates of
glutathione reductase and monitored by the appearance
of the TNB anion of DTNB [5,5\prime-dithiobis-(2-nitro-
benzoic acid)] at 412 nm. To assess the GSSG con-
centrations accurately, two perchloric acid extracts were
used, one with N-ethylmaleimide (NEM) to determine
GSSG so that preparative oxidation of small amounts of
GSH to GSSG was avoided. The second extract was
prepared in the absence of NEM to determine GSH,
which is present in amounts excess to GSSG.

Results are presented as mean ± SEM. The p values
were calculated using Student’s t test for both paired
and unpaired variates.
Results

Numerous investigators have demonstrated depressed maximal rates of phosphorylating respiration in mitochondria isolated from ischemic hearts. Likewise, the mitochondria prepared from ischemic hearts, and used in the present study, show significant decreases in ADP-stimulated respiration with either succinate or glutamate as respiratory substrate (Table 1). In general, reperfusion of the ischemic zone produces little or no additional effect on respiratory rates. A small, but statistically insignificant, increase in succinate-supported respiration is observed in the case of mitochondria prepared by polytron homogenization. As demonstrated in previous studies, verapamil pretreatment protects against ischemic-related respiratory decreases. With glutamate as substrate, this protection is statistically significant (Table 1). A trend toward reversal is observed with succinate, although these values are not different from either control or ischemic respiratory rates.

In contrast to generalized effects of ischemia on respiratory rates of both mitochondrial preparations, significant decreases in the stoichiometry of coupling ATP production to oxygen consumption are observed only in the "polytron" mitochondria (Table 1). These decreases are apparent with both substrates and are also present in mitochondria isolated following reperfusion of the ischemic zone. Only under one condition does the ADP:O ratio decrease significantly in mitochondria prepared by nagarse, i.e., reperfusion with succinate as substrate.

The effects of alterations in oxygen metabolism in the ischemic/reperfused myocardium are thought to reflect the process of cell death. In the mitochondria, protection against the onset of peroxidative events is provided by glutathione peroxidase with subsequent reduction of glutathione disulfide by glutathione reductase. Thus, changes in the oxidation-reduction levels of GSH and GSSG should be a sensitive indicator of oxidative stress in the mitochondria. The effects of reperfusion in the absence and presence of verapamil on glutathione levels and redox ratios are directly compared with the values obtained for mitochondria prepared from ischemic myocardium (Figure 1). Reperfusion in the absence and presence of verapamil tends to reverse the ischemic changes in GSH and GSSG toward levels found in control mitochondria. However, a significant increase (p<0.05) in GSH over ischemic values is observed only in polytron mitochondria prepared from control and verapamil-treated reperfused hearts. Conversely, and in both mitochondrial preparations, GSSG decreases significantly (p<0.05) to levels comparable to control in both the reperfused and verapamil-treated, reperfused groups (Figure 1A). No statistical differences are found for either GSH or GSSG between control and reperfused (± verapamil) groups. The GSH:GSSG ratios found for both preparations of mitochondria isolated from control and verapamil-treated hearts (and for the polytron preparation from reperfused hearts in the absence of verapamil) increase significantly (p<0.05) over ratios in ischemic mitochondria. When GSH:GSSG ratios are compared between mitochondria isolated from hearts reperfused in the absence and presence of verapamil, only the polytron mitochondria from verapamil-treated hearts demonstrate a significant elevation in the redox value, p<0.05 (Figure 1B).

The alterations in the reduction levels of glutathione following ischemia and reperfusion suggest that glutathione peroxidase may be acting to detoxify oxygen

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Table 1. Respiratory Rates and ADP/O Ratios: Mitochondria From Control, Ischemic, and Ischemic/Reperfused Hearts

<table>
<thead>
<tr>
<th>Group</th>
<th>Phosphorylating rates (ng atoms/min/mg)</th>
<th>Glutamate-malate</th>
<th>Succinate + rotenone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polytron mitochondria</td>
<td>Nagarse mitochondria</td>
<td>Polytron mitochondria</td>
</tr>
<tr>
<td>1 Control</td>
<td>315 ± 26</td>
<td>467 ± 36</td>
<td>319 ± 29</td>
</tr>
<tr>
<td>2 Ischemic</td>
<td>221 ± 37*</td>
<td>355 ± 40†</td>
<td>226 ± 17*</td>
</tr>
<tr>
<td>3 Ischemic reperfused</td>
<td>223 ± 42*</td>
<td>297 ± 51†</td>
<td>273 ± 65</td>
</tr>
<tr>
<td>4 Ischemic reperfused + verapamil</td>
<td>298 ± 30§</td>
<td>367 ± 34</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>ADP/O (ng atoms/min/mg)</th>
<th>Glutamate-malate</th>
<th>Succinate + rotenone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polytron mitochondria</td>
<td>Nagarse mitochondria</td>
<td>Polytron mitochondria</td>
</tr>
<tr>
<td>1 Control</td>
<td>2.76 ± 0.13</td>
<td>2.63 ± 0.2</td>
<td>1.63 ± 0.12</td>
</tr>
<tr>
<td>2 Ischemic</td>
<td>2.43 ± 0.11*</td>
<td>2.55 ± 0.11</td>
<td>1.26 ± 0.14*</td>
</tr>
<tr>
<td>3 Ischemic reperfused</td>
<td>2.31 ± 0.12*</td>
<td>2.61 ± 0.14</td>
<td>1.25 ± 0.27‡</td>
</tr>
<tr>
<td>4 Ischemic reperfused + verapamil</td>
<td>2.77 ± 0.15§</td>
<td>2.70 ± 0.2</td>
<td>1.68 ± 0.16‡</td>
</tr>
</tbody>
</table>

Results are expressed mean ± SEM.

*p < 0.001 with respect to Group 1; †p < 0.01 with respect to Group 1; ‡p < 0.02 with respect to Group 1; §p < 0.02 with respect to Group 3; ‡‡p < 0.05 with respect to Group 3 by Student's t test for paired and nonpaired data.
FIGURE 1. Effect of reperfusion and verapamil on mitochondrial gluthathione from ischemic heart. Panel A: Increases in mitochondrial contents of reduced glutathione (GSH) and decreases in oxidized glutathione (GSSG) are expressed with respect to measured levels of the corresponding species in mitochondria from ischemic hearts. Ischemic levels of GSH and GSSG for polytron and nagarse preparations of mitochondria are, respectively: 2.55 ± 0.55 nmol/mg, 4.61 ± 0.91 nmol/mg, and 0.044 ± 0.0037 nmol/mg, 0.0556 ± 0.0124 nmol/mg. Panel B: Increases in the glutathione oxidation-reduction levels in mitochondria compared with the ratios measured following ischemia. The GSH/GSSG values for polytron and nagarse preparations of ischemic mitochondria are 58.4 ± 13.0 and 93.0 ± 22.8.

abundance of verapamil (Table 2). Decreased phospholipid content in ischemia is reflected by decreases in cardiolipin (from 9.41 ± 0.6 to 7.48 ± 1.00 mol %) and phosphatidylcholine (from 37.22 ± 1.22 to 34.8 ± 1.6 mol %). With reperfusion, phosphatidyl-ethanolamine concentrations are significantly decreased in both preparations of mitochondria (Table 2). With the additional loss of phosphatidylethanolamine on reperfusion and no further effects on other phospholipid components, the mole fraction of cardiolipin in the ischemic/reperfused membrane is only slightly less than the fractional content present in control polytron mitochondria (8.7 mol %). Phosphatidylcholine increases slightly in fractional composition following reperfusion in both the polytron (37.22 ± 1.22 to 39.34 ± 0.016 mol %) and nagarse mitochondria (38.84 ± 0.85 to 40.94 ± 0.55 mol %).

The loss of diacylphospholipids with ischemia and reperfusion is not accompanied by any detectable change in concentrations of lyso phosphatidylcholine or lyso phosphatidylethanolamine. Since high lyso phospholipase activities are present in cardiac tissue, rapid degradation of the lyso phosphatides formed may obscure any associated rise in these levels due to phospholipase A2 activity. In contrast to ischemia and reperfusion, verapamil pretreatment maintains the total phospholipid content of the mitochondria at control levels. Although the cardiolipin composition of mito-
Table 2. Phospholipid Content in Mitochondria Isolated From Control, Ischemic, and Ischemic/Reperfused Hearts

<table>
<thead>
<tr>
<th>Polytron mitochondria (nmol lipid phosphate/mg protein)</th>
<th>Control</th>
<th>Ischemic</th>
<th>Reperfused</th>
<th>Reperfused + verapamil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phospholipids</td>
<td>298.2 ± 14.9</td>
<td>264.5 ± 27.9*</td>
<td>236.8 ± 16*</td>
<td>271.9 ± 9.9§</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>28.1 ± 3.6</td>
<td>19.8 ± 5.6*</td>
<td>20.6 ± 1.5†</td>
<td>23.3 ± 2.8</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>108.3 ± 8.0</td>
<td>105.8 ± 6.0</td>
<td>77.3 ± 3.8*</td>
<td>97.4 ± 6.8§</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>110.0 ± 7.2</td>
<td>92.1 ± 8.5†</td>
<td>93.2 ± 8.3†</td>
<td>112.2 ± 8.5§</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>13.1 ± 4.1</td>
<td>9.8 ± 6.7</td>
<td>13.7 ± 4.7</td>
<td>11.0 ± 2.1</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>9.9 ± 3.2</td>
<td>10.1 ± 4.4</td>
<td>9.0 ± 1.3</td>
<td>6.6 ± 3.7</td>
</tr>
<tr>
<td>Lyso phosphatidylethanolamine</td>
<td>10.7 ± 3.3</td>
<td>9.5 ± 3.9</td>
<td>7.8 ± 2.8</td>
<td>5.3 ± 3.9</td>
</tr>
<tr>
<td>Lyso phosphatidylcholine</td>
<td>7.3 ± 2.6</td>
<td>6.1 ± 2.1</td>
<td>7.5 ± 3.7</td>
<td>5.6 ± 4.5</td>
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</table>

<table>
<thead>
<tr>
<th>Nagarse mitochondria (nmol lipid phosphate/mg protein)</th>
<th>Control</th>
<th>Ischemic</th>
<th>Reperfused</th>
<th>Reperfused + verapamil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phospholipids</td>
<td>274.1 ± 13.5</td>
<td>262.7 ± 12.6</td>
<td>263.9 ± 25.9</td>
<td>270.5 ± 10.9</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>23.4 ± 2.4</td>
<td>22.7 ± 1.8</td>
<td>23.8 ± 3.5</td>
<td>24.5 ± 3.6</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>93.3 ± 4.3</td>
<td>90.8 ± 6.0</td>
<td>84.3 ± 2.94</td>
<td>94.9 ± 4.6§</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>106.5 ± 4.7</td>
<td>107.8 ± 4.1</td>
<td>108.1 ± 3.3</td>
<td>110.8 ± 3.8</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>10.9 ± 4.3</td>
<td>8.0 ± 3.7</td>
<td>12.8 ± 3.9</td>
<td>10.6 ± 3.0</td>
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<tr>
<td>Sphingomyelin</td>
<td>11.5 ± 3.3</td>
<td>7.8 ± 3.0</td>
<td>6.3 ± 2.4</td>
<td>7.3 ± 3.1</td>
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<tr>
<td>Lyso phosphatidylethanolamine</td>
<td>10.0 ± 2.6</td>
<td>8.1 ± 2.2</td>
<td>8.5 ± 3.7</td>
<td>7.6 ± 4.6</td>
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<tr>
<td>Lyso phosphatidylcholine</td>
<td>12.1 ± 2.6</td>
<td>10.0 ± 4.7</td>
<td>8.8 ± 4.1</td>
<td>7.3 ± 4.3</td>
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<td>Lyso phosphatidylcholine</td>
<td>7.8 ± 2.5</td>
<td>6.7 ± 4.9</td>
<td>5.6 ± 2.4</td>
<td>5.6 ± 4.5</td>
</tr>
</tbody>
</table>

Results are expressed mean ± SEM.
* p < 0.001 with respect to control; † p < 0.01 with respect to control; ‡ p < 0.05 with respect to control; § p < 0.01 with respect to reperfused; || p < 0.05 with respect to reperfused by Student's t test for paired and nonpaired data.

Concomitant with significant losses in phosphatidylethanolamine following reperfusion are the significant decreases in both stearic (18:0) and arachidonic (20:4) acids (Table 3). Since the predominant ethanolamine phospholipid in canine cardiac mitochondria corresponds to 18:0-20:4 diacylphosphatidylethanolamine,36 an overall decrease in the bilayer content of phosphatidylethanolamine should be paralleled by losses in its constituent fatty acids. Conversely, an increased frac-

Table 3. Fatty Acid Content in Mitochondria Isolated From Control, Ischemic, and Ischemic/Reperfused Hearts

<table>
<thead>
<tr>
<th>Polytron mitochondria (mole percent)</th>
<th>Control</th>
<th>Ischemic</th>
<th>Reperfused</th>
<th>Verapamil + reperfused</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 16:0</td>
<td>14.92 ± 0.65</td>
<td>14.41 ± 1.39</td>
<td>15.34 ± 0.80</td>
<td>16.08 ± 0.18</td>
</tr>
<tr>
<td>C 18:0</td>
<td>19.50 ± 0.26</td>
<td>19.39 ± 0.25</td>
<td>17.17 ± 0.944</td>
<td>18.38 ± 1.10</td>
</tr>
<tr>
<td>C 18:1</td>
<td>15.64 ± 0.87</td>
<td>17.31 ± 1.48</td>
<td>20.56 ± 0.84*</td>
<td>17.95 ± 1.16</td>
</tr>
<tr>
<td>C 18:2</td>
<td>26.72 ± 1.00</td>
<td>25.42 ± 1.29</td>
<td>26.27 ± 0.90</td>
<td>26.03 ± 1.27</td>
</tr>
<tr>
<td>C 20:4</td>
<td>23.47 ± 0.90</td>
<td>23.47 ± 1.07</td>
<td>20.66 ± 0.29†</td>
<td>21.55 ± 0.21§</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nagarse mitochondria (mole percent)</th>
<th>Control</th>
<th>Ischemic</th>
<th>Reperfused</th>
<th>Verapamil + reperfused</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 16:0</td>
<td>15.89 ± 0.36</td>
<td>13.03 ± 0.80†</td>
<td>17.16 ± 1.1</td>
<td>16.79 ± 0.62</td>
</tr>
<tr>
<td>C 18:0</td>
<td>19.31 ± 0.55</td>
<td>18.92 ± 1.02</td>
<td>17.06 ± 0.65‡</td>
<td>17.33 ± 0.54‡</td>
</tr>
<tr>
<td>C 18:1</td>
<td>16.05 ± 0.39</td>
<td>16.40 ± 1.16</td>
<td>19.95 ± 0.43*</td>
<td>17.17 ± 0.385§</td>
</tr>
<tr>
<td>C 18:2</td>
<td>25.56 ± 0.66</td>
<td>25.71 ± 1.84</td>
<td>26.7 ± 1.39</td>
<td>26.60 ± 0.87</td>
</tr>
<tr>
<td>C 20:4</td>
<td>23.22 ± 1.00</td>
<td>24.48 ± 1.00</td>
<td>19.15 ± 0.65†</td>
<td>22.11 ± 0.90</td>
</tr>
</tbody>
</table>

Results are expressed mean ± SEM.
* p < 0.01 with respect to control; † p < 0.02 with respect to control; ‡ p < 0.05 with respect to control; § p < 0.01 with respect to reperfused; || p < 0.05 with respect to reperfused by Student's t test for paired and nonpaired data.
tional content of phosphatidylcholine in mitochondria from reperfused myocardium also may be associated with increased content of oleic acid as observed in both preparations of mitochondria (Table 3). Pretreatment of the ischemic/reperfused hearts with verapamil significantly protects against the loss of arachidonic acid (and loss of phosphatidylethanolamine, Table 2) during reperfusion. A trend toward recovery of stearate is also seen in the polytron preparation from verapamil-reperfused hearts as is recovery of the control composition of oleate (Table 3).

Activation of mitochondrial phospholipase A₂ by Ca²⁺, concomitant with inhibition of acyl-CoA lysophospholipid acyltransferase, is associated with mitochondrial swelling and alterations in Ca²⁺ uptake and release pathways. To examine the relation between the observed decrease in the mitochondrial glutathione redox ratio and proposed alterations in mitochondrial membrane permeability in the presence of exogenous Ca²⁺, the effect of ischemia on mitochondrial Ca²⁺ uptake and release was determined. Following 60 minutes of ischemia, both the potential dependent uptake and the release of Ca²⁺ on the Na⁺–Ca²⁺ exchange carrier are decreased in both preparations of mitochondria (Table 4). These data and the demonstration of increased rates of ruthenium-red–insensitive Ca²⁺ efflux from ischemic mitochondria (Table 4) are consistent with a more permeabilized inner membrane following addition of exogenous Ca²⁺ and subsequent enhancement of phospholipase A₂ activity. These results are consistent with our earlier studies, using the same model of ischemic arrest, in which decreases in both Ca²⁺ uptake and Na⁺–Ca²⁺ exchange as well as premature release of accumulated Ca²⁺ are prevented by verapamil pretreatment.

Discussion

In these studies, verapamil pretreatment of the ischemic/reperfused myocardium protects against structural and functional abnormalities associated with 60 minutes of ischemia or ischemia and reperfusion. A protective effect of verapamil on mitochondrial ultrastructure has been previously demonstrated in hypoxic rabbit myocardium and in ischemic canine hearts. Functionally, the rates of phosphorylating respiration and coupling of ATP production to electron transport are preserved within the range of control values. Verapamil also attenuates the decreases in total (and specific) phospholipid content seen in mitochondria isolated from both the ischemic and reperfused hearts. The protective effects of verapamil also correlate with maintenance of glutathione redox ratios in the mitochondrial matrix.

Alterations in ischemic mitochondrial membrane phospholipids, fatty acid content and microviscosity have been studied in both pig and rat heart. In both studies, an increase in membrane microviscosity was seen following coronary occlusion independent of any change in phospholipid content. These membrane alterations were attributed to increased mitochondrial cholesterol content with no change in fatty acid composition or were attributed to increases in the percent saturated fatty acids in lysophosphatidylcholine while a reduction occurred with lysophosphatidylethanolamine. Although the cholesterol content of the isolated mitochondria was not examined, we are unable to demonstrate any changes in lysophospholipids in mitochondria from either ischemic or ischemic/reperfused hearts. It might be expected that phospholipase A₂ activation should result in increased production of lysophospholipids, which could accumulate if the rates of production exceed the rates of transacylation via the mitochondrial acyl-CoA:lysophospholipid transacylase. However, loss of mitochondrial diacylphospholipids in the absence of a change in the respective lysophospholipid levels does not obviate activation of phospholipase A₂ during ischemia or reperfusion. Two possibilities exist that can explain the lack of lysophospholipid accumulation seen in these studies. First, due to the amphiphilic nature of these compounds, they probably partition into the aqueous solvent phase during preparation and washing of the mitochondria. Secondly, the lysophospholipids formed may be released to either the mitochondrial matrix or cytosol where they are rapidly degraded as a result of high levels of cardiac lysophospholipase activities.

The decreases in phospholipid content observed in the present study approximate most closely those results from a three-hour occlusion study, which also employed a canine model of ischemia. In that report, total phospholipid content decreased an average of 15% with no changes in sphingomyelin content, suggesting a phospholipase-mediated pathway. We also demonstrated an ischemic-related decrease in total phospholipids, although the changes seen within one hour of ischemia (as opposed to the longer period in the prior study) are associated with specific alterations in cardiolipin and phosphatidylcholine levels only. Although

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**Table 4. Ca²⁺ Uptake and Release in Mitochondria Isolated From Control and Ischemic Hearts**

<table>
<thead>
<tr>
<th></th>
<th>Ca²⁺ (10 μM) uptake</th>
<th>RR-insensitive Ca²⁺ release</th>
<th>Na⁺–Ca²⁺ exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polytron mitochondria (ng ions/min/mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>43.3 ± 7.7</td>
<td>0.27 ± 0.16</td>
<td>5.66 ± 0.94</td>
</tr>
<tr>
<td>Ischemic</td>
<td>22.8 ± 3.9</td>
<td>1.49 ± 0.88</td>
<td>1.33 ± 0.91</td>
</tr>
<tr>
<td>Nagarse mitochondria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>48.1 ± 7.4</td>
<td>0.25 ± 0.15</td>
<td>5.58 ± 1.69</td>
</tr>
<tr>
<td>Ischemic</td>
<td>38.6 ± 8.1</td>
<td>0.51 ± 0.17</td>
<td>4.06 ± 0.36</td>
</tr>
</tbody>
</table>

Results are expressed mean ± SEM.
it is possible that these decreases in cardiolipin and phosphatidylcholine are mediated by phospholipase A₂, the mechanism by which loss of these phospholipids may occur may be more complicated than a generalized Ca²⁺-activated pathway. First, the exclusion of phosphatidylethanolamine as a substrate for hydrolysis during ischemia despite a bilayer distribution similar to cardiolipin (predominantly matrix side) is difficult to explain. Second, cardiolipin is a relatively poor substrate for phospholipase, yet this phospholipid is lost from the mitochondria during ischemia to a greater extent than is phosphatidylcholine (27 vs. 16%).

The molecular basis for the selective reperfusion-mediated decrease in phosphatidylethanolamine also is not known. However, differences do exist between the membrane localization and compartmentation of phosphatidylethanolamine and in its higher plasmalogen content when compared with phosphatidylcholine (20 vs. 1.9% respectively, Palmer et al¹⁰). Therefore, differences in the susceptibility of the various major phospholipid classes to Ca²⁺-activated hydrolysis may be dependent on the membrane location and proximity to endogenous phospholipase A₂ as well as the substrate suitability of the respective phospholipid molecules and their configuration in the bilayer.

The effect of reperfusion of the ischemic myocardium and the protective effects of verapamil on mitochondrial phospholipid content, fatty acid composition, and glutathione content have not been previously explored. The purpose of the present study is to identify a metabolic indicator that may be diagnostic of reperfusion injury and which may suggest a biochemical setting required for expression of these events. Thus, preferential excision of phosphatidylethanolamine during reperfusion, and not ischemia, is associated with decreases in mitochondrial stearate and arachidonate, the predominant fatty acid components of mitochondrial ethanalamiglycerophospholipids. The lack of any further effect of reperfusion on rates of mitochondrial phosphorylating respiration may be related to published studies based on marker enzymes, demonstrating no measured enzymatic dependency, e.g., rotenone-sensitive NADH-cytochrome C reductase or malate dehydrogenase, on pools of phosphatidylethanolamine that are readily accessible to phospholipase A₂. Therefore, mitochondrial function appears not to be deleteriously affected by the reperfusion protocol since no alterations in rates of phosphorylating respiration are seen when compared with mitochondria isolated following ischemia alone. Furthermore, our studies indicate that the changes in mitochondrial function during ischemia and reperfusion are not caused by the accumulation of high levels of lipid peroxidation products in the membranes. However, a lack of any measured elevation in these products is not altogether surprising. First, reactive oxygen products when formed may not be produced to such an extent that the protective mechanisms involving superoxide dismutase, catalase, and glutathione peroxidase are overwhelmed. Therefore, the result of oxidative processes could lead to a change in glutathione redox status in the absence of any observable lipid peroxidation. Alternatively, the lack of peroxidized lipids in the mitochondria could be explained by a relatively slow peroxidation of the phospholipids followed by normal, or enhanced, phospholipid degradation such that the peroxidation products do not accumulate to any measurable extent in the membranes. Finally, rather than an exacerbation of oxidative stress to the mitochondria, reintroduction of flow is associated with a trend toward restoration of reduced levels of matrix glutathione. Thus, if reperfusion is associated with increased levels of oxygen free radicals, as suggested by others, mitochondria isolated from the reperfused regions of the ischemic myocardium appear to be competent in their detoxification. In this regard, arachidonic acid at the SN-2 position of phosphatidylethanolamine may be particularly susceptible to reperfusion effects, for if the second mechanism involving phospholipid degradation applies (see above), a loss of this phospholipid during reperfusion could result from lipid peroxidation and excision via increased phospholipase A₂ activity.

In addition to the acceleration of phospholipase A₂ activity by increased levels of peroxidized fatty acids, it is also possible that Ca²⁺ entry during reperfusion may activate mitochondrial phospholipase A₂. Protection against the changes in phospholipid content by verapamil pretreatment may reflect the reported ability of verapamil to inhibit mitochondrial phospholipase A₂. The physiologic mechanism by which a net loss in membrane phospholipids due to deacylation is prevented involves reesterification of fatty acids via the enzyme acyl-CoA lysophospholipid acyltransferase. During oxidative stress, reacylation activity is inhibited as a result of the sulphydryl sensitivity of this enzyme. In the presence of extramitochondrial Ca²⁺, mitochondria that are oxidatively stressed (as seen by alterations in the matrix GSH:GSSG) undergo rapid swelling and permeability alterations due to activation of phospholipase A₂ with subsequent release of accumulated Ca²⁺. This response to a more oxidized glutathione ratio is also typified in the handling of Ca²⁺ by ischemic mitochondria as shown presently and previously by this laboratory. These results are in contrast to those obtained by Nayler et al in which isolated mitochondria from hypoxic hearts demonstrate stimulation of Ca²⁺ uptake. However, no information concerning release of accumulated Ca²⁺ is presented by these workers, nor is any data available on the glutathione content of these mitochondria.

In summary, the basic premise on which we base our conclusions is that the sulphydryl-dependent mechanism for maintenance of normal mitochondrial levels of diacylglycerophospholipids is inhibited during myocardial ischemia as a result of alterations in the matrix glutathione redox ratio. This alteration may then set up a sequence of metabolic events that is unique to reperfusion, i.e., specific loss of mitochondrial phosphatidylethanolamine. We have previously demonstrated that the GSH:GSSG is decreased by 85% in
mitochondria prepared from ischemic heart. In the present study, there is no statistically significant difference in either GSH or GSSG levels between control and verapamil-pretreated ischemic mitochondria. This is consistent with the preservation of phospholipid and fatty acid composition as well as mitochondrial function from these hearts. However, reperfusion of ischemic hearts also is associated with a trend toward restoration of GSH and GSSG levels (and thus the glutathione redox potential) to control levels. A pattern toward reestablishing synthesis of reduced glutathione likely results from a resupply of reducing equivalents to glutathione reductase during the reperfusion interval. In spite of the trend toward reduction of glutathione during reperfusion, the data suggest continued inability of reacetylation activity to keep pace with probable enhancement in phospholipase A₂. Therefore, in all likelihood, a fraction of the mitochondrial glutathione in ischemic and reperfused hearts is involved in glutathione-protein mixed-disulfide formation with persisting inhibited mitochondrial function from these hearts. However, reperfusion of ischemic hearts also is associated with a trend toward restoration of GSH and GSSG levels (and thus the glutathione redox potential) to control levels. A pattern toward reestablishing synthesis of reduced glutathione likely results from a resupply of reducing equivalents to glutathione reductase during the reperfusion interval. In spite of the trend toward reduction of glutathione during reperfusion, the data suggest continued inability of reacetylation activity to keep pace with probable enhancement in phospholipase A₂. Therefore, in all likelihood, a fraction of the mitochondrial glutathione in ischemic and reperfused hearts is involved in glutathione-protein mixed-disulfide formation with persisting inhibited synthesis of sulfhydryl-sensitive activities, including acyl-CoA:lyso phospholipid acyltransferase. While perfusion protocols that include specific inhibitors of phospholipase A₂, such as chloropromazine, probably would delay the loss of mitochondrial phospholipids in ischemia, effective restoration of control mitochondrial metabolism and structure may finally be dependent on return of sulfhydryl-dependent functional activities in the mitochondrial membrane-matrix space.

References


**Key Words:** ischemia/reperfusion • verapamil • glutathione • lipid hydroxy acids.
Protection by verapamil of mitochondrial glutathione equilibrium and phospholipid changes during reperfusion of ischemic canine myocardium.

K Kajiyama, D F Pauly, H Hughes, S B Yoon, M L Entman and J B McMillin-Wood

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