Brain Phospholipid Metabolism During Ischemia

BY FRANK M. YATSU, M.D.

Abstract: Molecular mechanisms accounting for the vulnerability of brain to ischemic damage are incompletely understood. To assess the role of membrane integrity, phospholipid metabolism is investigated in subcellular organelles of brain following varying degrees of ischemia. Global brain ischemia is produced in rabbits by combining hypotension and hypoxia (4% oxygen). Restoration of blood pressure and oxygen after a three-minute isoelectric EEG results in full recovery, while a five-minute isoelectric EEG results in either a lack of recovery or neurological deficits. Quantitative analysis of seven phospholipids in subcellular organelles shows no consistent pattern of phospholipid changes following ischemia. Incorporation of radioactive phosphate, on the other hand, discloses moderate alterations following five-minute ischemia. These findings indicate that brain ischemia provokes phospholipid changes, but their role in impairing membrane integrity and in initiating irreversible ischemic brain damage is uncertain.

Introduction

The molecular basis for the vulnerability of brain to hypoxia and ischemia is not completely understood. Various neurochemical investigations have implicated energy metabolism, neurotransmitter synthesis, ATPase activity and protein and lipid synthesis. Previous studies from this laboratory assessed alterations in whole brain lipids following hypoxia-ischemia and showed diminished synthesis of rapidly metabolized lipids, inositide glycerophosphatide and serine glycerophosphatide. The continued suppression of these constituents might contribute to the development of irreversible damage. Our present study investigated phospholipid metabolism in brain mitochondria, microsomes and synaptosomes during varying periods of brain ischemia. Phospholipid metabolism was assessed by both quantitative analysis and the incorporation of intracisternally injected (³²P) phosphate in each of the subcellular organelles. These studies were carried out in our rabbit model of ischemia.

The purposes of our studies were twofold: first, to identify a lipid which may play a role in determining the vulnerability of brain to ischemia; and second, to identify the subcellular organelle which receives the brunt of ischemic injury as reflected in structural lipid alterations.

Methods

PRODUCTION OF BRAIN ISCHEMIA

Cerebral ischemia was produced in awake, randomly bred adult New Zealand white rabbits by combining hypotension with hypoxia as previously reported. The degree of ischemic insult is gauged by the duration of an isoelectric electroencephalogram (EEG). After introduction of 100% oxygen and normalization of blood pressure, rabbits sustaining a three-minute isoelectric EEG uniformly recovered without detectable neurological deficits. On the other hand, rabbits allowed to sustain a five-minute isoelectric EEG did poorly despite circulatory restoration. The latter rabbits either did not recover significant EEG activities or after extubation were unable to hop or right themselves. This model provides an opportunity to assess varying degrees of ischemic insult to the brain and its ability to regain function after restoration of circulation.

SUBCELLULAR ISOLATION OF BRAIN

For analytical studies, the brain was removed as soon as craniotomy was performed. The brain was homogenized in 9 vols of ice-cold 0.32 M sucrose. The microsomal, mitochondrial and synaptosomal fractions were isolated by the method of Gray and Whittaker. From animals injected with labeled-phosphate, aliquots of subcellular fractions were counted for radioactivity before a lipid extraction. The protein contained in each fraction was determined by the method of Lowry et al.

INJECTION OF (³²P)-PHOSPHATE

One hour prior to the ischemic insult, 1 me of sodium (³²P)-Phosphate-HCl (New England Nuclear Corporation, Boston, Massachusetts) was injected intracisternally through the foramen magnum. Blood samples were drawn at various intervals from the cannulated femoral artery to assay serum radioactivity. Ninety minutes following the intracisternal injection of labeled phosphate, the brain was taken out. Portions (100 mg) of the anterior, middle and
posterior cerebral cortex were removed, weighed and counted for radioactivity. Liver slices were similarly prepared. For these purposes, the tissues were digested with 0.5 ml of NCS (Amersham/Searle), at 40°C overnight. To the clear suspension was added 0.1 ml of distilled water and 15 ml of PPO-POPOP* (Packard Instruments) and the samples were counted in a Beckman LS-233 liquid scintillation counter.

**LIPID ANALYSIS**

The mitochondria, microsomes and synaptosomes were extracted with 19 vols of chloroform-methanol (2:1, v/v) for one hour at room temperature. The insoluble protein was removed by filtration through a millipore filter and dried, and the radioactivity was counted. The extract was purified by the technique of Folch, Lees and Sloane Stanley. The upper phase obtained was dried under nitrogen and the radioactivity measured. Aliquots of the bottom phase, containing the total lipid fraction, were removed for weighing on a Cahn microbalance (Cahn Instruments, Paramount, California) and counted for radioactivity.

Seven separate phospholipids were isolated, identified and quantitated by the method of Rouser et al. The three lyso-phospholipids of ethanolamine, choline and serine glycerophosphatide were isolated, but because of their fractional quantities are not included in tabular form. The method involves two dimensional thin-layer chromatography. Phospholipids were quantitated by phosphorus determinations. Recoveries for phospholipids averaged 100%. The radioactivity of each phospholipid was determined after isolation on thin-layer chromatograms. The silicic acid spots containing the phospholipids were scraped into acid spots containing the phospholipids were scraped into

**Results**

The protein content of subcellular fractions varied from preparation to preparation, regardless of experimental conditions. Attempts for quantitative recovery of subcellular fractions, however, were not made. The yield of lipid per milligram protein was determined in one pair of control and five-minute ischemic synaptosomes. From 1.3 gm wet weight of five-minute ischemic brain, the synaptosomal fraction yielded 9.2 mg protein and 6.4 mg lipid. Despite the similar yield of lipid per milligram protein under control and experimental conditions, the possibility that ischemia may have altered the buoyancy of affected subcellular organelles cannot be excluded. Thus, altered lipid content of vulnerable organelles would prevent their recovery using customary sedimentation gradients.

Quantitative values of seven phospholipids of brain mitochondria, microsomes and synaptosomes from control, three-minute ischemic and five-minute ischemic brains are given in table 1 in microgram phosphorus per milligram lipid. Phospholipid

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**Footnotes:**

*PPO: 5-diphenyloxazole. POPOP: 1, 4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene.

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**Table 1:**

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Control</th>
<th>Three-Minute</th>
<th>Five-Minute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingomyelin</td>
<td>2.48</td>
<td>1.62</td>
<td>1.06</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>2.28</td>
<td>1.44</td>
<td>0.91</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>1.50</td>
<td>0.92</td>
<td>0.60</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>0.72</td>
<td>0.42</td>
<td>0.36</td>
</tr>
<tr>
<td>Lysophosphatidylethanolamine</td>
<td>0.26</td>
<td>0.12</td>
<td>0.07</td>
</tr>
<tr>
<td>Lysophosphatidylcholine</td>
<td>0.30</td>
<td>0.16</td>
<td>0.12</td>
</tr>
<tr>
<td>Lysophosphatidylserine</td>
<td>0.22</td>
<td>0.11</td>
<td>0.08</td>
</tr>
</tbody>
</table>

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*SNP = standard error of the mean. Numbers are given for each experimental period.*

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*Note: *PPO = 5-diphenyloxazole; POPOP = 1, 4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene.
recovery or the sum of each phospholipid is compared to the phosphorus content of an aliquot of the total lipidd fraction weighed to constant weight. Quantitative increase in phospholipids following severe ischemia is unaccountable on the basis of de novo synthesis. An explanation is the known loss of brain fatty acids during ischemia accounting for a fractional reduction in total lipids which gives an apparent rise in phospholipids.

Results on the relative incorporation of radioactively labeled (32P) in the four most highly labeled phospholipids — inositol glycerophosphatide, phosphatidic acid, choline glycerophosphatide, and ethanolamine glycerophosphatide — accounting for over 90% of the radioactivity from the subcellular organelles of control, three-minute and five-minute ischemic brains are presented in table 2. Alteration in the three-minute ischemic brain is a relative reduction in the labeling of ethanolamine glycerophosphatide in the microsomal fraction. In five-minute ischemic brain, the most abundant phospholipids, choline glycerophosphatide and ethanolamine glycerophosphatide, were reduced in all three subcellular fractions while phosphatidic acid and/or inositol glycerophosphatide were enhanced. Radioactivity in the anterior, middle, and posterior portions of the cerebral cortex varied from experiment to experiment. This indicated variable access of labeled phosphate to the subarachnoid space following intracisternal injection and rendered comparative analysis of specific activities invalid. Total radioactivity in the liver constituted less than 5% of the total administered dose of labeled phosphate. Serial determination of serum activity showed a persistent, high level of radioactivity throughout the time period of the experiment. Although the specific form of labeled phosphate in serum, i.e., lipid or phosphorylated intermediates, was not determined, this characteristic of serum labeling rendered "pulse labeling" experiments impractical.

Discussion

Neurochemical investigations assessing the exquisite vulnerability of brain to ischemia or hypoxia have focused principally upon energy metabolism, glycolysis, protein and lipid synthesis, neurotransmission and lysosomal activities. Specific experimental techniques used to produce cerebral ischemia include decapitation, exsanguination, vascular occlusion or embolism and strangulation. Each of these has methodological limitations. Our technique of creating global cerebral ischemia provides the opportunity of correlating neurological impairment with neurochemical changes. Our present studies explored the role of phospholipids as a monitor of membrane integrity. Phospholipid metabolism was specifically investigated by quantitative phospholipid analysis and by the patterns of labeled phosphate incorporation.
BRAIN PHOSPHOLIPID METABOLISM DURING ISCHEMIA

Following three-minute and five-minute ischemia, no consistent change in the pattern of phospholipids or in a single constituent is seen using analytical methods (table 1). For example, with three minutes of global brain ischemia, a decrease in serine glycerophosphatides and an increase in diphosphatidyl glycerol occur in both mitochondria and microsomes, and inositol glycerophosphate falls in mitochondria and synaptosomes. Choline glycerophosphate rises in microsomes and synaptosomes and in the latter, sphingomyelin increases and ethanolamine glycerophosphate decreases. After five minutes of cerebral ischemia, serine glycerophosphate is the single lipid showing similar rises in all three subcellular organelles. In microsomes and synaptosomes, sphingomyelin and diphosphatidyl glycerol both increase. A rise of phosphatidic acid occurs in microsomes, and ethanolamine and phosphatidic acid glycerophosphatides increase in mitochondria.

Increase in micrograms phospholipid per milligram total lipid following five-minute ischemia in both mitochondria and synaptosomes is not expected to represent net increase due to de novo synthesis. The increase, for example, of phospholipid from mitochondria with control and five-minute ischemia is 25.64 µg and 31.32 µg or an increase of 22%. Similarly for synaptosomes, the increase of phospholipid per milligram total lipid from control and five-minute ischemia is 27.06 µg to 29.44 µg or a 9% increase. This magnitude of phospholipid increase is not anticipated from previous studies on lipid synthesis following ischemia and hypoxia. A more likely explanation for the apparent increase in phospholipids is a decrease in the total lipid fraction. For example, loss of brain lipids, such as fatty acids, due to ischemia will reduce the total lipid fraction; the release of brain fatty acids during ischemia has been reported. Bazan reported the free fatty acid levels at approximately 40 µg per gram brain tissue immediately following ischemia created by decapitation. Within five minutes, the level rose fivefold to 200 µg per gram brain tissue. The possibility in our experiments of fatty acid loss accounting for an apparent increase in phospholipids with five-minute ischemia minimizes their quantitative changes as seen in table 1.

Incorporation of labeled phosphate into phospholipids was used to investigate their metabolism in membranes of brain mitochondria, microsomes and synaptosomes. Friedel and Schanberg have shown that intracisternally injected labeled-phosphate, the label was incorporated into phospholipids. For example, Cotman and Matthews find that the cortical energy charge of the adenylate pool to normalize quickly after the circulatory restoration, and isolated brain mitochondria show unimpaired synthesis of ATP. These results indicate that energy-dependent processes, rather than energy-synthesizing machinery, are particularly vulnerable to ischemia. That membrane in integrity is such a vulnerable, energy-dependent process is possible, but our data do not support a firm conclusion. Alterations of phosphatidic acid and inositol glycerophosphate metabolism in five-minute ischemic synaptosomes suggest another possible role of phospholipids in ischemic damage. A relationship between neurotransmission and inositol glycerophosphate metabolism has been known and DeRobertis strengthened this coupling by demonstrating a link between acetylcholine and inositol glycerophosphate in the "receptor protein." In addition, the
demonstration by Williams and Grossman of alterations confined to the cortical synaptic terminals during hemorrhagic shock provides morphological evidence implicating neurotransmission as a vulnerable focus of ischemia. These two findings plus a greater relative labeling of phosphatidic acid over inositol glycerophosphatide in synaptosomal membrane suggest a possible relationship between ischemic brain injury and defective neurotransmission. It is concluded from our studies that nonspecific phospholipid changes are seen following brain ischemia. Alterations of phosphatidic acid and inositol glycerophosphatide synthesis seen after severe, five-minute ischemia warrant further investigations.

References


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