31P Nuclear Magnetic Resonance Studies of Phosphoglyceride Metabolism in Developing and Degenerating Brain: Preliminary Observations


Abstract. 31P nuclear magnetic resonance (NMR) studies were conducted on perchloric acid extracts of the brain of one control, two Huntington’s disease (HD), one probable Alzheimer’s disease (AD), and one AD patient. These studies demonstrated significant elevations (over control) in the levels of phosphomonoesters in all brain areas of the patients with HD and AD even in areas devoid of neuropathological findings. Elevations of phosphodiesters were also observed, but they tended to reflect the degree of neuropathological change. We postulate that the 31P NMR findings represent molecular alterations with corresponding metabolic correlates which either antedate or occur in the absence of changes in cellular morphology or structure. As such the 31P NMR findings may reflect a subcellular “molecular neuropathology.”

Key Words: Degenerative disease; Phospholipids; 31P nuclear magnetic resonance.

INTRODUCTION

Recent 31P nuclear magnetic resonance (NMR) studies on mammalian brain reported from this laboratory have demonstrated: high levels of a phosphomonoester resonance (1); previously overlooked metabolic relationships in brain and brain slice preparations (1, 2); a differential response to anoxia in aged versus adult animals (3); and a two- to three-fold elevation of phosphomonoester resonances in neurodegenerative diseases such as Huntington’s disease (HD) and Alzheimer’s disease (AD) (4–6). The purpose of this report is to demonstrate changes in the content of phosphomonoesters and closely related biochemical derivatives in developing and degenerating brain, and to compare these changes with the neuropathologic findings in AD and HD. We suggest that the high levels of phosphomonoesters observed in developing brain reflect active membrane synthesis. We further suggest that the elevated phosphomonoester and phosphodiester levels found in AD and HD brains may reflect aberrant attempts by the diseased brains to regenerate membranes. Finally, we postulate that the NMR findings represent molecular changes and metabolic correlates that either antedate or occur in the absence of alterations in cellular morphology or structure.

From the Laboratory of Neurophysics, Departments of Psychiatry and Neurology, University of Pittsburgh, Pittsburgh, Pennsylvania (JWP, NJM); Department of Physiology and the Nuclear Magnetic Resonance Laboratory, Chicago College of Osteopathic Medicine (SJK, TG, JMF, JPT); and Department of Neurological Science, Rush-Presbyterian St. Lukes Medical Center, Chicago, Illinois (MMC).

Correspondence to: J. W. Pettegrew, M.D., Laboratory of Neurophysics, Departments of Psychiatry and Neurology, University of Pittsburgh, Western Psychiatric Institute and Clinic, 3811 O’Hara Street, Pittsburgh, PA 15213.

Supported in part by a grant from the United Cerebral Palsy Foundation, No. R-322-84, NIA grants 1 R01 AG05657-01 and AG05133-01 A1, Clinical Research Center grant No. MH30915, and NIH grant No. MH/NS 31862.
MATERIALS AND METHODS

Animals

Albino New Zealand rabbits of random sex and designated ages were used for these studies: newborn (N = 5, less than four hours (h) of age), neonates (N = 5, 36–48 h of age), ten-day-olds (N = 6), and 110-day-olds (N = 2). The neonates, newborn, and ten-day-old rabbits came from four litters; the 110-day-old rabbits were unrelated. The newborn, neonate, and ten-day-old rabbits were killed by total immersion into liquid nitrogen (N₂) until frozen solid. The 110-day-old rabbits were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneal), a tracheostomy performed, and the animals ventilated with 100% O₂ using a small animal respirator (ca. 30/minute) before and during freezing of the cranium under liquid-N₂ conditions in all animals.

Human Brain

Frozen human brain samples were obtained from Dr. Edward D. Bird, Director of the National Institute of Aging (NIA)-funded Brain Tissue Resource Center at McLean Hospital, Belmont, Massachusetts. Demographic and neuropathologic data are given in Table 1. The results of neuropathological examinations were provided by Dr. Bird.

Perchloric Acid Extraction

The frozen brain cortex from each animal was weighed and pulverized in a liquid-N₂-cooled mortar and pestle. The resultant tissue powder was transferred to a liquid-N₂-frozen polymuller centrifuge tube containing a standard aliquot of 60% perchloric acid (0.25 v/w) and extracted as described previously (1, 2). The extract was concentrated by lyophilization and purged of polyvalent cations by passing the sample through a potassium Chelex-100 column before analysis by ³¹P NMR. The human brain samples were extracted in a manner identical to that used to extract the rabbit brain samples.

³¹P NMR Spectroscopy

The NMR spectrometer system used in this investigation was either a Nicolet NT-200 equipped with deuterium stabilization or a Chemagnetics CMC-200LS. Both instruments are equipped with variable temperature, proton broad-band decoupling, and Fourier-transform capabilities and both operate at 81 MHz for ³¹P. The 1.0 ml brain extract samples in 20% D₂O were placed in 12 mm NMR microcell assemblies for NMR analysis. The samples were analyzed at 23°C under bi-level proton decoupling conditions so as to maintain a constant nuclear Overhauser enhancement while spinning the sample at 24 Hz to enhance signal resolution. The ³¹P chemical shift data are reported relative to the usual standard of 85% orthophosphoric acid. Chemical shifts follow the International Union of Pure and Applied Chemistry convention and are reported in the field independent units of δ (parts per million, or ppm). Details relating to the qualitative and quantitative ³¹P NMR analysis have been reported previously (1, 2, 7). The methodologic basis for the application of ³¹P NMR to the analysis of tissues and their extracts has been described elsewhere (8).

RESULTS

Brain cortical levels of a prominent phosphomonoester fall from mean levels of 6.4 ± 0.3 micromoles (µmol)/g wet weight (wt) in the newborn rabbit to mean levels of 1.7 ± 0.2 µmol/g wt at 110 days of age. The levels of cortical phosphomonoesters in the adult animal then remain stable and, as previously reported, are not affected by insults such as ischemia or hypoxia (Fig. 1 and Table 2) (1, 2). Recently, the prominent phosphomonoester in mammalian brain as detected by ³¹P NMR has been identified as phosphoethanolamine (2, 9).

The levels of phosphomonoesters and phosphodiesters are also elevated in autopsy
<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Sex</th>
<th>*Time to $-20^\circ$C (hours)</th>
<th>Neuropathological diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>M</td>
<td>6½</td>
<td>Frontal and parietal cortical sections showed neuronal loss in layers 1, 2, 3. No fibrillary astrocytosis and no neurofibrillary tangles or plaques. Caudate nucleus and dorsal putamen atrophic; marked depletion of small neurons and fibrillary gliosis was present. Neuropathological diagnosis: Huntington’s disease.</td>
</tr>
<tr>
<td>63</td>
<td>M</td>
<td>24</td>
<td>Cortical sections showed rare neuritic plaques; no neurofibrillary tangles. Amygdala, thalamus, mammillary body, caudate, putamen, globus pallidus, substantia innominata, and nucleus of Meynert normal. Hippocampus contained numerous neuritic plaques and neurofibrillary tangles, mostly in the Sommer sector; endplate gliosis. Neuropathological diagnosis: Probable Alzheimer’s disease.</td>
</tr>
<tr>
<td>62</td>
<td>M</td>
<td>5½</td>
<td>Cortical sections showed numerous shrunken neurons with eosinophilic cytoplasm and dark nuclei. No neuritic plaques or neurofibrillary tangles. Medial caudate nuclei showed loss of small neurons and fibrillary astrocytosis. Cerebellum had segmental loss of Purkinje cells, moderate Bergmann gliosis and necrosis of granular layer. Neuropathological diagnosis: Huntington’s disease and terminal encephalopathy.</td>
</tr>
<tr>
<td>65</td>
<td>M</td>
<td>13½</td>
<td>Cortical sections showed numerous neuritic plaques and neurofibrillary tangles. Hippocampus contained numerous plaques, tangles, and neurons with granulovacuolar degeneration; endplate gliosis; decreased pyramidal neurons. Numerous neurons of the basal nucleus of Meynert contained pink-staining cytoplasmic globules. Neuropathological diagnosis: Alzheimer’s disease.</td>
</tr>
</tbody>
</table>

* Time from death to storage of brain at $-20^\circ$C.

Brain samples from one patient with probable Alzheimer’s disease (AD), one patient with AD, and two patients with Huntington’s disease (HD) when compared to a control brain (Fig. 2) (4–6). The increased levels of phosphomonoesters and phosphodiester are seen in all areas of the brains in AD and HD, even in areas that are histologically normal, such as the cerebellum. The phosphomonoester elevation also appears more pronounced in the AD brain than in the HD brain, and in both diseases the phosphomonoester and phosphodiester elevations seem greatest in the areas of greatest neuropathological changes. The results given in Table 3 are for the individual phosphomono- and phosphodiester components. In Table 3, the most apparent alteration is an elevation in phosphoethanolamine (PE) in all sampled areas of diseased brain. The results given in Table 4 are expressed as the total integrated areas of the phosphomono- and phosphodiester regions since this degree of spectral resolution is what is currently seen with in vivo $^{31}$P NMR spectroscopy. Once again, phosphomonoesters are elevated in all areas of diseased brain (especially in AD), and the phosphodiester elevations are greatest in those areas of diseased brain with the most neuropathological changes, such as hippocampus and frontal cortex in the AD, and caudate nucleus in HD.
MATURATIONAL CHANGES IN ENERGY COMPOUNDS AND MEMBRANE CONSTITUENTS

Fig. 1. High-resolution (4.7 Tesla) $^{31}$P NMR spectra of neonatal and adult rabbit cortex. In the left lower corner the spectrum of a perchloric acid (PCA) extract is shown for the brains of neonatal animals. In the right upper corner is the spectrum for a PCA extract for the brains of adult animals. The phosphoethanolamine (PE) content is also plotted for animals of several different ages (<4 h, 36–48 h, 10 days, 110 days). There is a three-fold drop in PE content and an increase in PCR/Pi ratio upon brain maturation from the newborn to the adult. ATP: adenosine triphosphate. GPC: glycerol 3-phosphocholine. GPE: glycerol 3-phosphoethanolamine.

DISCUSSION

In postmortem mammalian brain, the components of the phosphomonoester resonance region have been identified as phosphoethanolamine, $\alpha$-glycerophosphate, and phosphocholine. Phosphoethanolamine is an anabolic precursor to phosphatidylethanolamine and a catabolic product of sphingomyelin. $\alpha$-Glycerophosphate is an anabolic precursor to phosphatidylethanolamine, phosphatidylcholine, and phosphoinositides. Phosphocholine is an anabolic precursor to phosphatidylcholine and sphingomyelin and a catabolic product of sphingomyelin. In the phosphodiester resonance region there are two major components, glycerol 3-phosphoethanolamine, a catabolic product of phosphatidylethanolamine and plasmalogen, and glycerol 3-phosphocholine, which is a catabolic product of phosphatidylcholine (10).

The phospholipid composition of the adult human cerebral cortex is 37% phosphatidylethanolamine, 35% phosphatidylcholine, 13% phosphatidylserine, 11% sphingomyelin, and 4% phosphatidylinositol. Therefore, $\alpha$-glycerophosphate, phosphoethanolamine, and phosphocholine are found only in the anabolic pathway of 89% of the phospholipids and are found in the anabolic (phosphocholine) as well as catabolic (phosphoethanolamine and phosphocholine) pathway of 11% (sphingomyelin). Therefore, to a first approximation, the phosphomonoester resonances quan-
TABLE 2
Rabbit $^{31}$P NMR of Brain Cortex Extracts

<table>
<thead>
<tr>
<th>Age</th>
<th>Phosphoethanolamine content micromoles/g wet weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;4 hours</td>
<td>6.4 ± 0.3</td>
</tr>
<tr>
<td>36–48 hours</td>
<td>5.3 ± 0.4</td>
</tr>
<tr>
<td>10 days</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>110 days</td>
<td>1.7 ± 0.2</td>
</tr>
</tbody>
</table>

titatively reflect phospholipid anabolic activity. The phosphodiester, glycerol 3-phosphoethanolamine, and glycerol 3-phosphocholine are found only in catabolic pathways. With these considerations in mind, the phosphomonoester/phosphodiester ratio approximates grey matter anabolic/catabolic activity. Recent studies we have done using the Fischer 344 rat from newborn to two years of age confirm that $^{31}$P NMR spectroscopy can monitor the brain phospholipid anabolic/catabolic flux activity as a function of development and aging (unpublished data).

We have previously demonstrated an elevated phosphomonoester resonance in rapidly dividing neuroblastoma clonal lines (11), and in a preliminary report, have recently shown the elevation of a prominent phosphomonoester resonance in developing and degenerating brain (12). These previous studies and the present one are in agreement with earlier studies that demonstrated a relative abundance of phosphoethanolamine in developing rabbit brain (13). A relatively prominent phosphomonoester resonance exhibiting the appropriate $^{31}$P chemical shift also has been reported in human neonatal brain (14, 15) and childhood neuroblastoma (16) in vivo using a surface coil technique.

Phosphomonoesters (phosphoethanolamine, phosphocholine, $\alpha$-glycerophosphate) are metabolically related to brain membrane phospholipid metabolism (10, 17). Therefore, a relative elevation of phosphomonoesters might be anticipated under conditions of increased membrane phospholipid synthesis, such as occurs in developing brain, in neural tumors, and, as the present findings suggest, in degenerating brain as well. The elevation of specific phosphomonoesters could also represent a relative enzymatic block in the metabolic pathway distal to the accumulating phosphomonoester.

The elevated levels of phosphomonoesters and phosphodiesters in AD and HD brains may represent an underlying derangement in membrane phospholipid metabolism. The phosphomonoester elevation may be the more sensitive indicator of early metabolic alterations since the phosphomonoester elevation occurs even in areas of brain devoid of discernible neuropathological abnormalities. Although the major contribution to the phosphomonoester resonance in AD and HD brains is probably phosphoethanolamine ($\delta = 3.84$), smaller contributions could be from $\alpha$-glycerophosphate ($\delta = 4.29$) and phosphocholine ($\delta = 3.33$) (Fig. 2). In contrast, the elevations in the phosphodiesters glycerol 3-phosphoethanolamine and glycerol 3-phosphocholine tend to reflect the degree of neuropathological involvement, e.g., for HD, the elevations were greatest in the caudate nucleus followed by the frontal cortex and normal in cerebellum; for AD, the elevations were greatest in the hippocampus followed by frontal cortex followed by caudate nucleus and cerebellum (Tables 3, 4). The elevation in phosphomonoester levels, therefore, appear to antedate cellular structural changes and may reflect increased phospholipid synthesis.
TABLE 3
Percent of Total PCA Extractable Phosphate in Huntington’s Disease (HD) and Control Brains Assayed by $^{31}$P NMR

<table>
<thead>
<tr>
<th></th>
<th>Cerebellum</th>
<th>Frontal A9</th>
<th>Caudate</th>
<th>Cerebellum</th>
<th>Frontal Brodmann area 9</th>
<th>Caudate</th>
</tr>
</thead>
<tbody>
<tr>
<td>αGP</td>
<td>2.2</td>
<td>3.6</td>
<td>7.3</td>
<td>3.0</td>
<td>2.6</td>
<td>3.5</td>
</tr>
<tr>
<td>PE</td>
<td>11.3</td>
<td>11.1</td>
<td>8.9</td>
<td>5.0</td>
<td>5.8</td>
<td>5.8</td>
</tr>
<tr>
<td>PC</td>
<td>2.9</td>
<td>3.5</td>
<td>1.6</td>
<td>4.1</td>
<td>2.2</td>
<td>4.6</td>
</tr>
<tr>
<td>Pi</td>
<td>68.1</td>
<td>60.2</td>
<td>64.0</td>
<td>77.7</td>
<td>72.3</td>
<td>76.4</td>
</tr>
<tr>
<td>GPE</td>
<td>3.0</td>
<td>5.7</td>
<td>7.0</td>
<td>2.0</td>
<td>3.3</td>
<td>2.8</td>
</tr>
<tr>
<td>GPC</td>
<td>4.3</td>
<td>3.7</td>
<td>4.4</td>
<td>1.3</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Total</td>
<td>91.8</td>
<td>87.8</td>
<td>93.2</td>
<td>93.1</td>
<td>88.2</td>
<td>93.4</td>
</tr>
</tbody>
</table>

αGP, α-glycerophosphate; PE, phosphoethanolamine; PC, phosphocholine; Pi, inorganic orthophosphate; GPE, glycerol 3-phosphoethanolamine; GPC, glycerol 3-phosphocholine; PCA, perchloric acid.

The phosphodiester elevations appear to correspond to the cellular changes and may reflect increased phospholipid turnover.

The AD case with neuropathological changes noted only in the hippocampus is important, as it demonstrates that the $^{31}$P NMR-detected abnormality was diffuse, even though the neuropathological abnormalities were limited to one area (hippocampus) of the brain. These findings suggest that $^{31}$P NMR can detect alterations in phospholipid metabolism before an alteration in cellular structure is evident by current neuropathological methods. The $^{31}$P NMR may also provide a metabolic correlate to the neuropathological findings. Alternatively one could argue that this is not a case of AD, since the neuropathological changes are limited to the hippocampus, and therefore, the abnormal $^{31}$P NMR findings are of questionable significance. However, there is not unanimity of opinion as to the quantitative or even qualitative neuropathological criteria for AD. One point of view contends that the neuropathological diagnosis of AD requires the presence of neurofibrillary tangles and neuritic plaques throughout the neocortex, in quantities greater than found with normal aging.

However, other points of view contend that the diagnosis of Alzheimer’s disease can be made with neuropathological changes only in the hippocampus (18) and can be made with the presence of only neuritic plaques and without neurofibrillary tangles (19). The latter points of view are broadly based on the clinical observation that dementia is the salient feature of the disease, and therefore, hippocampal involve-

Fig. 2. High resolution (4.7 Tesla) $^{31}$P NMR spectra of perchloric acid extracts from control, Alzheimer’s disease (AD) and Huntington’s disease (HD) cerebella. The high-energy phosphates (phosphocreatine, ATP, etc.) have all been converted to inorganic orthophosphate (Pi). The phosphomonoesters such as α-glycerophosphate (αGP), phosphoethanolamine (PE), and phosphocholine (PC) and the phosphodiesters such as glycerol 3-phosphoethanolamine (GPE) and glycerol 3-phosphocholine (GPC) are elevated in AD and HD brains even in areas with normal histology, such as the cerebellum.
| Percent of Total PCA Extractable Phosphate in Alzheimer's (AD), Huntington's (HD) and Control Brains Assayed by $^{31}$P NMR |
|---|---|---|---|---|---|---|
| Phosphomonoesters | Inorganic orthophosphate | Phosphodiester | Total |
| Control | Frontal | Brodmann area 9 | Caudate | Cerebellar cortex | Frontal | Brodmann area 9 | Caudate | Hippocampus | Cerebellar cortex | Frontal | Brodmann area 9 | Caudate | Hippocampus | Cerebellar cortex |
| Frontal | 10.6 | 72.3 | 76.4 | 77.7 | 5.4 | 3.4 | 3.1 | 3.3 | 9.1 ± 2.9 |
| Alzheimer's brain | 12.2 ± 1.6 | 57.6 | 70.4 | 64.5 | 6.2 ± 1.3 | 7.5 | 5.1 | 7.0 | 96.9 ± 1.4 |
| Alzheimer's hippocampus | 19.8 | 25.8 ± 6.2 | 70.4 | 64.5 | 5.0 | 5.1 | 3.3 | 98.5 |
| Total | 22.9 | 34.2 ± 3.7 | 56.0 ± 3.4 | 6.4 | 9.4 ± 3.2 | 99.8 |

The individual values and the mean ± SD are given.

PCA, perichloric acid.
ment alone (18) or neuritic plaques alone (19) could explain the clinical findings, and furthermore, the neuropathology in the early stages of the disorder may be more localized. This, however, does not mean that molecular or metabolic alterations giving rise to neuropathological changes in some areas of the brain could not be diffuse throughout the brain.

The findings reported herein are consistent with a number of studies over recent years which demonstrated alterations in the structural composition and function of neural membranes attendant to normal aging in animals (20–31). Support for an alteration in membrane phospholipids in HD comes from several independent studies. Alterations in membrane structure and biophysical parameters have been demonstrated in HD erythrocytes (32–35), lymphocytes (36, 37), and fibroblasts (38–41). In addition, more recent studies have demonstrated alterations in the biophysical structure (42–44) and function (45–50) of membranes of extraneural tissues obtained from patients with AD. However, this is apparently the first demonstration of a possible alteration in the anabolic pathway of phospholipid metabolism detectable in autopsy brain from AD patients as previously reported (4, 5).

Further evidence for chemically altered membranes in HD and AD comes from earlier neurocytological studies. Graveland et al (51), using a Golgi impregnation method, showed degenerative and regenerative changes in Huntington’s neostriatal spiny neurons. Scheibel (52), also using the Golgi impregnation method, referred to “lawless secondary growth” along the dendritic and somal membrane of AD neurons. Such presumably regenerative attempts must be associated with augmented membrane phospholipid synthesis.

This is a preliminary report of an on-going, more comprehensive study, which will be reported separately. Results to date comparing different brain areas have been obtained on 48 autopsy brain samples from six neuropathologically verified Alzheimer's disease patients, and 19 autopsy brain samples from five histologically verified control patients. These grouped results to date confirm the findings reported herein of elevated phosphomonoester resonances in Alzheimer’s brain (p < 0.001 by Student t-test; unpublished observations). We believe the $^{31}$P NMR findings of elevated phosphomonoesters in developing brain and phosphomonoesters and phosphodiester in HD and AD brain represent increased synthesis and turnover of membrane phospholipids, which is normal in the developing brain, but abnormal in HD and AD. These findings do not appear to be a postmortem artifact, because we have previously demonstrated no correlation between the interval from death to perchloric acid extraction and the levels of phosphomonoesters or phosphodiesters detected in brain (53). Similarly, in the present study the intervals between death and freezing the brains at −20°C do not appear to correlate with the phosphomonoester or phosphodiester levels (Tables 1, 4). Another report has also recently demonstrated findings similar to those reported herein in AD autopsy brain by $^{31}$P NMR spectroscopy (54). Further, detailed in vitro NMR studies should help elucidate these metabolic derangements and provide a rational basis for the use of in vivo $^{31}$P NMR spectroscopy as a clinical tool to investigate phospholipid metabolism in normal and diseased brain. The possibility now exists that $^{31}$P NMR spectroscopy may detect a “molecular neuropathology” in diseased brain tissue even before morphological changes become evident.

ACKNOWLEDGMENTS

The authors thank Dr. Edward D. Bird, Director of the McLean Hospital Brain Tissue Resonance Center, for providing the brain samples, and Ms. Mary Solarczyk for secretarial assistance.

REFERENCES


(Received 11 July 1986/Accepted 29 October 1986) MS86-72