

In Vitro ^{31}P NMR Spectroscopy Detects Altered Phospholipid Metabolism in Alzheimer's Disease

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ABSTRACT: In order to study possible metabolic derangements in Alzheimer's disease (AD), we performed phosphorus 31 nuclear magnetic resonance (^{31}P NMR) spectroscopy on brain samples obtained at autopsy from 7 patients with AD and 9 control subjects. Aqueous solutions of brain tissue contained well-defined peaks of intermediate compounds in phospholipid metabolism, including the phosphomonoesters phosphocholine and phosphoethanolamine, and the phosphodiester glycerophosphorylcholine and glycerophosphorylethanolamine. ^{31}P NMR spectra also displayed the inorganic phosphorus signal, which provides an index to the *in vivo* concentration of high-energy compounds.

We found evidence for altered phospholipid metabolism in that relative levels of phosphomonoesters were decreased, and phosphodiester increased, in frontal and parietal regions of patients with AD compared to control subjects. The inorganic phosphorus resonance peaks were similar in AD and control subjects, suggesting that energy stores are not diminished in AD. These preliminary data are consistent with the hypothesis that abnormalities in phospholipid metabolism contribute to possible neuronal membrane dysfunction and impaired cholinergic neurotransmission in AD.

RÉSUMÉ: La spectroscopie par RMN au ^{31}P *in vitro* détecte des changements du métabolisme des phospholipides dans la maladie d'Alzheimer. Nous avons procédé à l'examen spectroscopique par résonance magnétique nucléaire au ^{31}P (RMN ^{31}P) d'échantillons de cerveaux provenant de l'autopsie de 7 patients atteints de maladie d'Alzheimer (MA) et de 9 sujets témoins, dans le but d'étudier d'éventuelles perturbations métaboliques dans la MA. Des solutions aqueuses de tissu cérébral contenaient des pics bien définis correspondant à des composés intermédiaires du métabolisme des phospholipides, comprenant les phosphomonoesters, la phosphocholine et la phosphoéthanolamine et les phosphodiester, la glycérphosphorylcholine et la glycérphosphoryléthanolamine. Le signal correspondant au phosphore inorganique, qui fournit un index de la concentration des composés à haute énergie *in vivo*, était également présent dans les spectres obtenus par RMN ^{31}P .

Nous avons trouvé des indices qui nous portent à croire qu le métabolisme des phospholipides est altéré: la baisse des niveaux relatifs de phosphomonoesters et l'augmentation des phosphodiester dans la région frontale et pariétale des patients atteints de MA lorsqu'ils sont comparés aux sujets témoins. Les pics de résonance correspondant au phosphore inorganique étaient semblables chez les patients atteints de MA et les sujets témoins, suggérant que les réserves d'énergie ne sont pas diminuées dans la MA. Ces données préliminaires sont comptables avec l'hypothèse qui veut que les anomalies du métabolisme des phospholipides contribuent à une dysfonction éventuelle de la membrane du neurone et à une altération de la neurotransmission cholinergique dans la MA.

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Proton nuclear magnetic resonance (NMR) brain imaging is well established in medical practice as a valuable adjunct to the diagnosis of numerous brain diseases, including the dementias due to Alzheimer's disease (AD), stroke, and Creutzfeldt-Jakob disease.¹⁻¹⁰ MR images are equally as accurate as x-ray computerized tomography (CT) scans in detecting brain abnormalities, and are superior to CT scans in displaying the morphology of structures such as the hippocampus that are particularly affected in AD, and in detecting small cerebral infarcts and other subcortical white matter lesions.¹¹ Despite these clinical advantages of MR scans, neither they nor CT brain scans reveal pathognomonic features of AD.¹² Thus, the pathophysiologic changes that result in abnormal cerebral function in AD are not revealed by anatomic images of the brain.

In order to circumvent these limitations and gain additional insight into possible metabolic derangements that characterize AD, we have begun to obtain NMR spectra of phosphorus-containing compounds in brains obtained at postmortem examination from patients with AD and from nondemented control subjects. NMR spectroscopy has been used for the past 30 years to study the chemical and physical structure of isolated compounds, but its application to biological tissue is new. Among the various nuclei with potential for study, phosphorus 31 (³¹P) had the most appeal and allowed us to address two possible mechanisms producing cell death and causing AD: diminished energy stores, and altered biochemical composition of neuronal membranes.

MATERIALS AND METHODS

Brains were obtained at autopsy from 7 patients with histologically verified AD, and 9 non-demented control subjects. The mean age for the AD group was 75.6 years (range 61 to 90) and 67.8 years (range 28 to 91) for the control subjects. The mean interval between death and autopsy was 18.8 hours (range 5 to 48) for the AD group and 25.8 hours (range 3 to 103) for the control group. Brains were frozen in liquid nitrogen and stored at -70°C. Aqueous solutions of brain samples from frontal, parietal, temporal, and occipital brain regions for NMR analysis were prepared by the perchloric acid (PCA) extraction method of Glonek et al.¹³ Briefly, the frozen brain samples were pulverized to a powder in a steel mortar that was chilled in liquid nitrogen. A solution of 10% PCA (1 ml/g brain tissue) was added dropwise to the powder and this mixture was centrifuged at 17,000 RPM for 10 minutes. The supernatant was removed, kept cool in ice, and the pH was adjusted to 9.0 with KOH. The resultant salt precipitate was removed during a second centrifugation at 10,000 RPM for 5 minutes. The supernatant was frozen, lyophilized, and reconstituted in a solution of 20 mM EDTA and 20% D₂O; the pH was adjusted to 9.0.

³¹P NMR spectra were generated on a Bruker HX270 spectrometer interfaced with a Nicolet computer housed at the Bitter National Magnet Laboratory at the Massachusetts Institute of Technology (Director, Dr. Leo Neuringer). The spectrometer is equipped with deuterium field stabilization, computer controlled temperature, proton band-decoupling, and Fourier transformation capability. The brain extract sample was placed in a 10 mm diameter NMR tube with a flat bottom, and analyzed at 34°C while spinning. Spectra were generated with a one-pulse sequence with the pulse width corresponding to a 45° tip angle, 0.1 second interpulse delay, spectral width of 5,000 Hz,

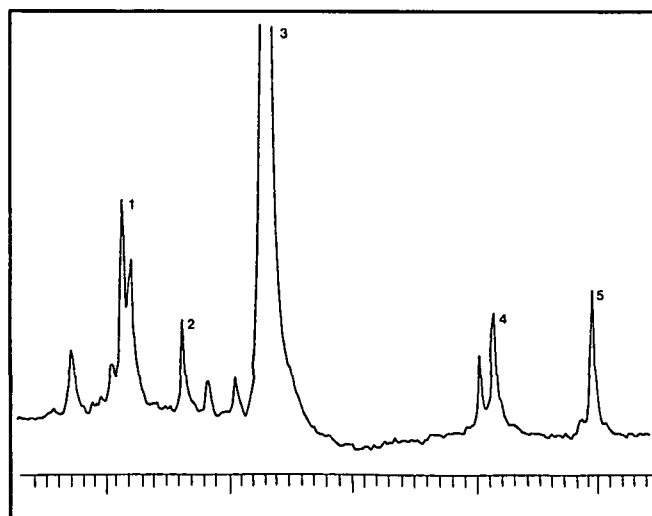


Figure 1 — ³¹P NMR spectrum from the frontal lobe of a non-demented control subject. The pertinent peaks are: 1 = phosphoethanolamine; 2 = phosphocholine; 3 = inorganic phosphate; 4 = glycerophosphoryl-ethanolamine; 5 = glycerophosphorylcholine. The unmarked peaks are inconsistent and not identified.

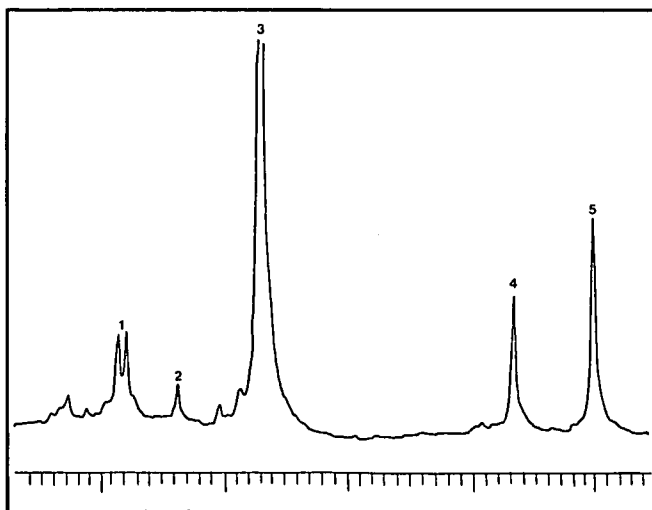


Figure 2 — ³¹P NMR spectrum from the frontal lobe of a patient with Alzheimer's disease. The peaks are numbered as in Figure 1. The PM peaks (1 and 2) are decreased, and the PD peaks (4 and 5) are increased, compared to the spectrum from the control subject.

4096 data points, and 8192 acquisitions per spectrum. The spectral peak positions were reported according to the International Union of Pure and Applied Chemistry Convention of field-independent units of "delta" (parts per million). ³¹P chemical shift data were reported relative to the conventional standard of 85% inorganic orthophosphate at 0.00 ppm. The GPC resonance was used as an internal standard reference because of its relatively constant ³¹P chemical shift of 0.13 ppm which is not influenced by variable pH, ionic strength, or counterion conditions.¹⁴

Each compound's relative concentration was based upon the area under its resonance peak. For purposes of analysis, the individual value for each compound was expressed as a ratio compared to the total estimated phosphorus signal (Pi + Pe + Pc + GPE + GPC). Data were analyzed by the two-tailed t-test for unpaired samples. Each compound was analyzed comparing AD vs. controls.

Table 1: Ratios of inorganic phosphorus (Pi), phosphomonoesters (PM), and phosphodiester (GPC, GPE) to total phosphorus content (T) in brains of patients with AD and normal control subjects.

BRAIN REGION		n	Pi/T	PM/T	GPC/T	GPE/T	GPC/GPE	PD/T
Frontal	NL	7	81.4±2.1	11.5± 1.7	3.2± .3	2.7± .2	1.2 ±.1	5.9± .5
	AD	7	81.2±2.1	6.9± .7*	6.2±1.6	4.6± .5*	1.3 ±.3	10.8±1.9*
Parietal	NL	3	84.8±3.3	9.9± 2.6	2.3± .8	2.1± .6	1.0 ±.2	4.5±1.4
	AD	2	79.4±1.9	7.0± .4	7.1± .7	6.2±1.2	1.2 ±.3	13.2±1.5*
Temporal	NL	4	82.3±3.6	10.9± 1.4	4.0±1.6	2.3± .7	1.5 ±.3	6.3±2.3
	AD	3	82.3±3.6	27.7±20.9	6.8±2.3	3.9± .8	1.66±.3	10.7±3.1
Occipital	NL	3	85.5±2.5	10.6± 3.1	1.7± .3	2.1± .7	.99±.3	3.8± .9
	AD	1	78.2	7.3	5.6	6.9	.82	12.5

PMs are significantly decreased in the frontal lobe, and PDs are significantly increased in frontal and parietal lobes, of patients with AD. Concentrations of Pi, PMs, and PDs do not differ significantly across brain regions within the AD and within the control groups.

*p<.05

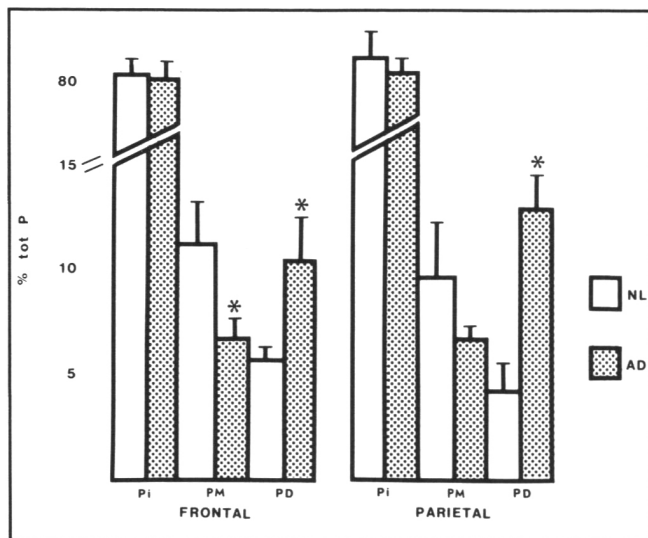


Figure 3 — Relative concentrations of Pi, PMs, and PDs in aqueous extracts of frontal and parietal brain tissue from patients with AD (stippled bars) and normal control subjects (open bars). Concentrations are expressed as a percent of the total phosphorus signal. The PMs are significantly reduced ($p<.05$) in frontal lobe and PDs significantly increased ($p<.05$) in frontal and parietal lobes of patients with AD.

RESULTS

³¹P NMR spectra (Figures 1 & 2) from aqueous solutions of brain tissue contained multiple peaks, including those of prime interest: inorganic phosphate (Pi); the phosphomonoesters (PMs), phosphoethanolamine (Pe) and phosphocholine (Pc); and the phosphodiester (PDs), glycerophosphorylethanolamine (GPE) and glycerophosphorylcholine (GPC). These signals were observed in all brain samples subjected to ³¹P NMR spectroscopy, and were highly reproducible. As expected, signals corresponding to high-energy phosphate compounds, such as ATP and phosphocreatine (PCr), disappeared after death.

Within patient groups, there were no significant differences in Pi, PMs, or PDs across brain regions (Table 1). Thus, spectra from frontal regions did not differ from spectra obtained from parietal regions in AD. Similarly, there were no differences among brain regions in control subjects. Between subject groups however, there were significant differences. Compared to con-

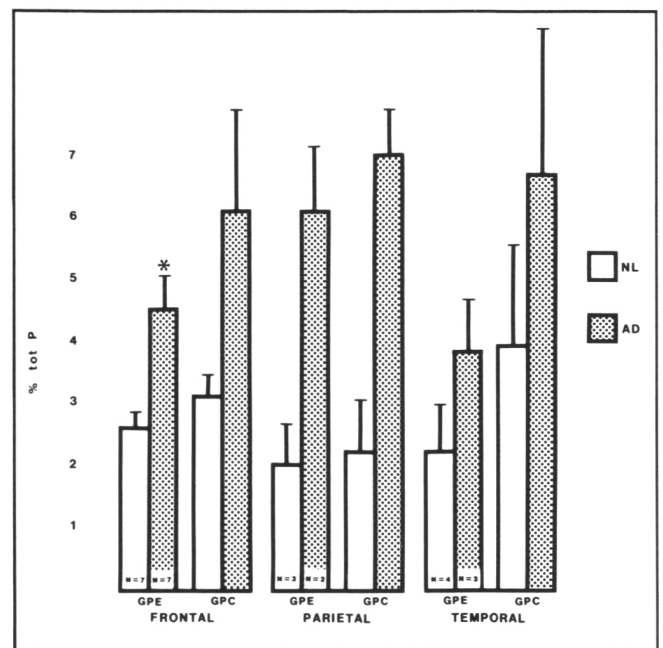


Figure 4 — Relative concentrations of the individual PDs, GPE, and GPC in aqueous extracts of frontal, parietal, and temporal lobes in patients with AD (stippled bars) and normal control subjects (open bars). There is a significant increase in the GPE: total phosphorus ratio in the frontal lobe of patients with AD; other increases were not statistically significant due to the small number of samples and their large variance.

rol brains, there was a significant ($p<.05$) reduction in the concentration of PMs in the frontal lobe in AD and a significant ($p<.05$) increase in PDs in both frontal and parietal regions (Figure 3). Analysis of the individual PDs indicated that although GPC and GPE increased in all regions of AD brain, the only significant increase ($p<.05$) was in GPE concentration in the frontal lobe (Figure 4). Although relative levels of PDs were significantly increased in AD, levels of GPC and GPE were tightly yoked and there was no evidence for dissociations within PD metabolism in either AD or control brains. Thus, the ratio of GPC to GPE did not differ significantly in AD or in controls, and was close to 1.0 in frontal, parietal, temporal, and occipital regions in both groups of subjects. No significant differences were observed in Pi concentrations between AD and control brains.

DISCUSSION

The results of this study indicate that application of ^{31}P NMR spectroscopic techniques to postmortem brain tissue can provide insights into biochemical abnormalities that characterize AD, especially those involving the phospholipids (PLs) that constitute a major portion of neuronal membranes and contribute to acetylcholine (ACh) metabolism. During normal aging, the amount of brain PLs decreases,¹⁵ but few data are available regarding probable PL abnormalities in AD. This dearth of information is surprising because PLs are involved in aspects of brain function thought to be affected in neurodegenerative diseases such as AD, including neuronal membrane composition, fluidity, and neurotransmitter receptor numbers,¹⁶ and ACh synthesis.¹⁷ Our data are consistent with previous studies¹⁸⁻²⁷ indicating that ^{31}P NMR spectroscopy could detect PMs and PDs, and that their concentrations were relatively unaffected by ischemia, hypoxia, or death. Although ATP and PCr resonances disappear from ^{31}P NMR spectra after death, compounds such as Pc, Pe, GPC, and GPE are stable; they reflect PL metabolism, and changes in their levels provide biochemical markers of cerebral degenerative processes. Outside of the central nervous system, GPC levels were found to be increased in muscle disease, perhaps accompanied by altered feedback inhibition of the enzyme lysolecithinase.²⁸⁻²⁹ Findings similar to these prompted Burt and Ribolow's²⁸ hypothesis that an increase in PDs may be taken as a reliable marker of altered membrane PL composition. Our results are consistent with that hypothesis, and suggest that alterations in GPC and GPE together characterize membrane dysfunction.

In general, Pe and Pc are precursors and GPE and GPC are catabolites in the cycle of phosphatidylcholine (PC) biosynthesis, although the synthetic pathways are reversible. Studies on the synthetic and degradative enzymes that catalyze these actions are just beginning and will further clarify the relationships among these compounds and the relative importance of each pathway to overall PC and PL synthesis. For example, the activity of phospholipase D, the enzyme that catabolizes PC hydrolysis to release free choline, is reduced in brains of patients with AD to an extent comparable to that of choline acetyltransferase activity.³⁰ This relationship suggests one of two possibilities: either that phospholipase D is specifically located in cholinergic nerve terminals, such that its decreased activity reflects their degradation, or that the decrease in phospholipase D activity reflects a general abnormality of PL metabolism in AD. The increase in GPC described in this report and in one previous study²⁷ might reflect a compensatory increase in PC degradation in brains lacking adequate phospholipase D activity. The reduction in PMs in AD may occur as a result of impaired synthesis. It may also result from accelerated PM utilization in order to resynthesize the PLs that are apparently degraded faster than normal, as evidenced by increased levels of PDs.

Our observation that PMs are decreased and PDs increased in AD must be considered preliminary because we have examined only a few brain regions in a small number of cases. Our data differ somewhat from the 2 previous reports of *in vitro* ^{31}P NMR spectroscopic studies in AD. Pettegrew et al²⁷ analyzed ^{31}P NMR spectra based upon postmortem brain samples obtained from 3 patients: classical AD, atypical AD (with histopathologic lesions limited to the hippocampus), and a normal control subject. These investigators reported that Pc and Pe levels were twice

as high in AD as in the normal subject, and levels of GPC and GPE were one and a half times higher. They interpreted these findings as being consistent with an elevation of phospholipase activity. The NMR changes were present throughout the brain of the atypical AD case, suggesting that ^{31}P NMR spectroscopy detects metabolic alterations prior to any demonstrable histopathological change. Our finding that PD levels are increased in AD agrees with the results of Pettegrew et al and are consistent with an abnormality of phospholipase activity, perhaps in the catabolic pathway of PC. In similar ^{31}P NMR spectroscopic studies, Barany et al³¹ reported that the ratio of GPC to GPE was 0.28 in brains of 9 normal control subjects, and 0.84 in 9 AD patients. These investigators did not specify the brain regions examined; we found ratios of GPC to GPE close to 1.0 in frontal, parietal, temporal, and occipital lobes. In contrast to our report and that of Pettegrew et al, Barany et al found no change in Pc and Pe levels in AD. The discrepancies between our results and those of Barany et al are difficult to explain, because the extraction and spectroscopic procedures were identical in the three studies. Additional studies with more tissue samples than currently examined will be necessary to clarify these differences.

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