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Evidence for a membrane defect in Alzheimer disease brain

(phospholipid metabolism/neurodegeneration/Down syndrome/Huntington disease/Parkinson disease)

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ABSTRACT **To determine whether neurodegeneration in Alzheimer disease brain is associated with degradation of structural cell membrane molecules, we measured tissue levels of the major membrane phospholipids and their metabolites in three cortical areas from postmortem brains of Alzheimer disease patients and matched controls. Among phospholipids, there was a significant ($P < 0.05$) decrease in phosphatidylcholine and phosphatidylethanolamine. There were significant ($P < 0.05$) decreases in the initial phospholipid precursors choline and ethanolamine and increases in the phospholipid deacylation product glycerophosphocholine. The ratios of glycerophosphocholine to choline and glycerophosphoethanolamine to ethanolamine were significantly increased in all examined Alzheimer disease brain regions. The activity of the glycerophosphocholine-degrading enzyme glycerophosphocholine cholinephosphodiesterase was normal in Alzheimer disease brain. There was a near stoichiometric relationship between the decrease in phospholipids and the increase of phospholipid catabolites. These data are consistent with increased membrane phospholipid degradation in Alzheimer disease brain. Similar phospholipid abnormalities were not detected in brains of patients with Huntington disease, Parkinson disease, or Down syndrome. We conclude that the phospholipid abnormalities described here are not an epiphenomenon of neurodegeneration and that they may be specific for the pathomechanism of Alzheimer disease.**

Brain lesions characteristic of Alzheimer disease (AD) include amyloid deposition, the formation of neurofibrillary tangles, and neuronal degeneration. The etiology and pathophysiology of neuronal death in AD are unknown. Cell membrane lipid abnormalities have been described in AD brain, and it has been hypothesized that these contribute to amyloid deposition (1, 2) and neuronal dysfunction (3). Initial evidence for a biochemical abnormality in the metabolism of phospholipids came from *in vitro* ^{31}P NMR spectroscopic studies showing that the ratio of glycerophosphocholine (GPC) to glycerophosphoethanolamine (GPE) as well as levels of glycerophosphodiesters and phosphomonoesters were increased in AD brain (4–6). Quantitative HPLC analysis has demonstrated that the increase in glycerophosphodiesters was due to accumulation of the phospholipid catabolites GPC and GPE (7, 8). The biochemical mechanisms accounting for the increase of phospholipid catabolites are unknown. To investigate the abnormalities in the phospholipid metabolic pathways, we examined levels of the parent phospholipids, their precursors, and their catabolites (see Fig. 1) in three cortical brain areas obtained at autopsy from AD patients and matched controls. To rule out the possibility that the elevation in GPC reflects a slowing in its degradation, we measured GPC cholinephosphodiesterase activity. Furthermore, we compared levels of phospholipid catabolites in brains of patients with AD and controls to those in Down syndrome, Parkinson disease, and Huntington disease.

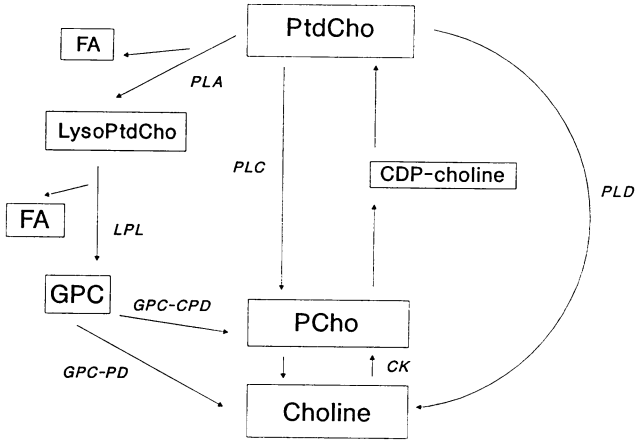

FIG. 1. Diagram of phosphatidylcholine (PdtCho) metabolism: Intermediates and enzymes. FA, fatty acid; *PLA*, phospholipase A; *LPL*, lysophospholipase; *PLC*, phospholipase C; *PLD*, phospholipase D; *GPC-CPD*, GPC cholinephosphodiesterase; *GPC-PD*, GPC phosphodiesterase; *CK*, choline kinase; PCho, phosphocholine; CDP, cytidine diphosphate. Identical biochemical pathways exist for phosphatidylethanolamine (PtdEtn).

EXPERIMENTAL PROCEDURES

Subjects and Brain Tissues. Brain tissue samples of 10 AD patients were pairwise matched with 10 neurologically normal control subjects according to age at death, postmortem time interval, and storage time at −80°C (Table 1). All AD patients met clinical (9) and neuropathological (10) criteria for the diagnosis of AD; control subjects did not have the clinical or neuropathological diagnosis of AD.

At autopsy, frontal (Brodmann area 45/46), primary auditory (Brodmann area 41/42/43), and parietal (Brodmann area 7B) cortices were frozen on dry ice. Autopsy samples of AD patients and control subjects as well as samples from two individuals with Down syndrome and three additional patients with Parkinson disease were obtained from the Tissue Resource Center at Massachusetts General Hospital. Frontal lobe cortex (Brodmann area 45/46) from three individuals with Down syndrome and five patients with Huntington disease were obtained from the McLean Hospital Brain Bank.

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Abbreviations: AD, Alzheimer disease; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine.

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Table 1. Characteristics of brain tissue samples

Diagnosis	*n* Total	♀	♂	Age at death, years	Postmortem interval, hr
Control	10	3	7	74.4 ± 4.6 (38–89)	10.3 ± 1.0 (5–17)
AD	10	5	5	75.7 ± 3.6 (51–95)	9.0 ± 1.3 (4–16)
HD	5	3	2	63.2 ± 4.0 (52–71)	9.8 ± 0.9 (8–12)
DS	5	3	2	52.2 ± 6.7 (36–69)	17.0 ± 2.4 (11–23)
PD	3	0	3	78.5 ± 3.7 (73–83)	12.7 ± 5.3 (2–18)

Values are means ± SEM; ranges are given in parentheses. There were no statistical differences between control, AD, Huntington disease (HD), and Parkinson disease (PD) groups. Age of Down syndrome (DS) patients differed ($P < 0.05$) from all other groups and the postmortem interval of Down syndrome differed ($P < 0.05$) from control, AD, and Huntington disease groups.

Tissue Extraction. Approximately 200 mg (wet weight) of cortical grey matter was dissected on a cold plate at −15°C and weighed after removal of remaining meninges, blood vessels, and white matter. Tissue was extracted according to Folch *et al.* (11). Aqueous and organic phases were dried by vacuum centrifugation and stored at −70°C. The interphase pellets were air-dried.

Phospholipid Assay. Dry organic phases were reconstituted in chloroform/methanol (1:1, vol/vol) and phospholipids were separated by silica gel chromatography (Analtech Uniplate, Pre Adsorbent 250 μm) using chloroform/ethanol/triethylamine/water (30:34:30:8, vol/vol) as the mobile phase. Phosphatidic acid comigrated with phosphatidylinositol in this system. Ethanolamine plasmalogen comigrated with PdtEtn; sums of both phospholipids were referred to as PtdEtn. Individual phospholipid bands were hydrolyzed with 70% (wt/vol) perchloric acid at 190°C for 1 hr, and phospholipid phosphorus was measured photometrically (12).

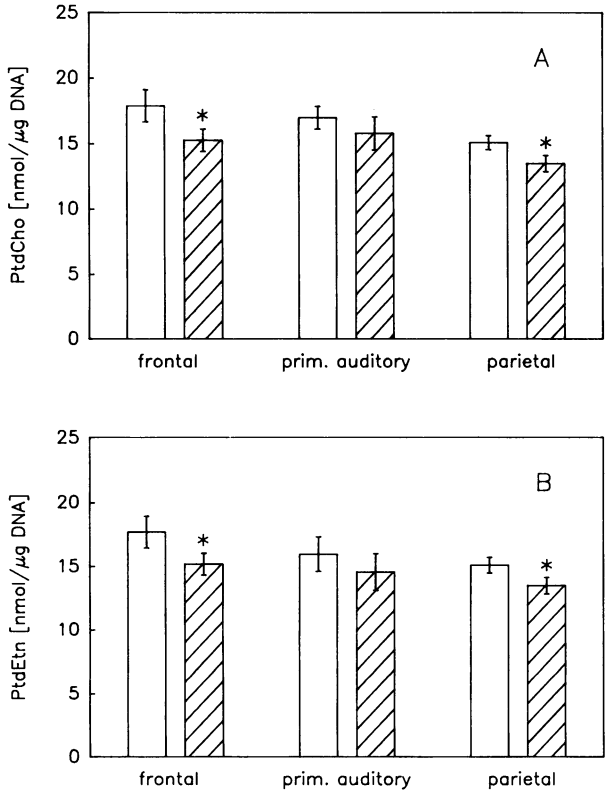

FIG. 2. Phospholipid levels in human brain cortex: PtdCho (*A*) and PdtEtn (*B*) in controls (open bars) and AD (hatched bars). Bars represent means ± SEM. *, $P < 0.05$ versus respective control (one-sided *t* test). $n = 10$ for each area and group.

Non-Choline-Containing Water-Soluble Phospholipid Metabolites. The dry aqueous phases were reconstituted in 87.5 mM $NaHCO_3$, pH 8.0, and derivatized with an equal volume of 2 mM 9-fluorenylmethyl chloroformate in dry acetone. Derivatives were separated by HPLC using a Microsorb C-18 column (Rainin Instruments) and detected fluorimetrically (8).

Choline-Containing Water-Soluble Phospholipid Metabolites. GPC, phosphocholine, and choline were separated by HPLC according to Liscovitch *et al.* (13). Phosphocholine fractions were hydrolyzed by using alkaline phosphatase (Sigma) at 7 units/ml in 100 mM glycylglycine, pH 10.7, containing 10 mM $MgCl_2$ and 20 mM $ZnSO_4$, at 37°C for 45 min, followed by choline assays. GPC fractions were hydrolyzed with 6 M hydrochloric acid at 90°C for 1 hr, prior to choline assay. Standard curves for choline-containing metabolites were constructed in parallel with each set of analyses.

Choline Assay. Choline as well as hydrolysates of choline-containing metabolites was quantified by HPLC with an on-line choline oxidase reactor column (Bioanalytical Systems, West Lafayette, IN) and electrochemical detection (14). The mobile phase consisted of 50 mM Na_2HPO_4, pH 8.5. All AD samples were measured in duplicates and as pairs or triplets together with the matched normal controls and neurologically diseased tissues in parallel to circumvent differences in recovery rates.

Ratios. GPC-to-choline and GPE-to-ethanolamine ratios were calculated for each individual sample, using the absolute amounts of these compounds in each sample.

GPC Cholinephosphodiesterase (EC 3.1.4.38) Assay. [^{14}C]GPC was made from L-dipalmitoyl Ptd[*methyl*-^{14}C]Cho (New England Nuclear) by alkaline hydrolysis, and purity was monitored by HPLC (13). Brain tissue samples were homogenized in 20 vol of ice-cold 0.25 M sucrose and incubated with excess [^{14}C]GPC (1.3 mM, 70,500 dpm/mmol) as substrate in a 70 mM glycine buffer, pH 9.5, at 30°C (15). The reaction was stopped with ice-cold methanol. Phospho[^{14}C]choline in the aqueous phase of chloroform/water (2:1, vol/vol) extracts were quantified by HPLC (13) using a Berthold LB 506 C radioactivity detector. Specific GPC cholinephosphodiesterase activity was calculated from the amount of phospho[^{14}C]choline formed from [^{14}C]GPC per tissue protein and incubation time. Total protein in the homogenates was determined by using the Pierce protein assay.

DNA Assay. Air-dried interphase pellets of the Folch extracts were dissolved in 0.3 M NaOH at 60°C for 2 hr and ultrasonicated. Total tissue DNA was determined fluorimetrically (16). Concentrations of phospholipids and metabolites were expressed per μg of DNA. Tissue levels of DNA were approximately 1 μg/mg of tissue (wet weight) and did not differ in any of the examined groups. Thus, similar results were obtained when tissue weight was used as denominator.

Table 2. Levels of phospholipids in human brain cortex

Area	Group	*n*	Phospholipid, nmol/μg of DNA PtdSer	Sphingomyelin	PtdIns/PA
Frontal	Control	10	6.68 ± 0.60	3.28 ± 0.43	0.85 ± 0.11
	AD	10	6.03 ± 0.50	3.11 ± 0.25	1.62 ± 0.14
Prim. aud.	Control	10	6.68 ± 0.70	1.70 ± 0.20	0.985 ± 0.20
	AD	10	6.35 ± 0.72	1.63 ± 0.17	0.854 ± 0.09
Parietal	Control	10	5.22 ± 0.18	2.60 ± 0.19	1.06 ± 0.09
	AD	10	4.83 ± 0.19	2.55 ± 0.22	1.04 ± 0.07

Prim. aud., primary auditory cortex; PtdSer, phosphatidylserine; PtdIns/PA, sum of phosphatidylinositol and phosphatidic acid. There were no significant differences between AD and respective control groups.

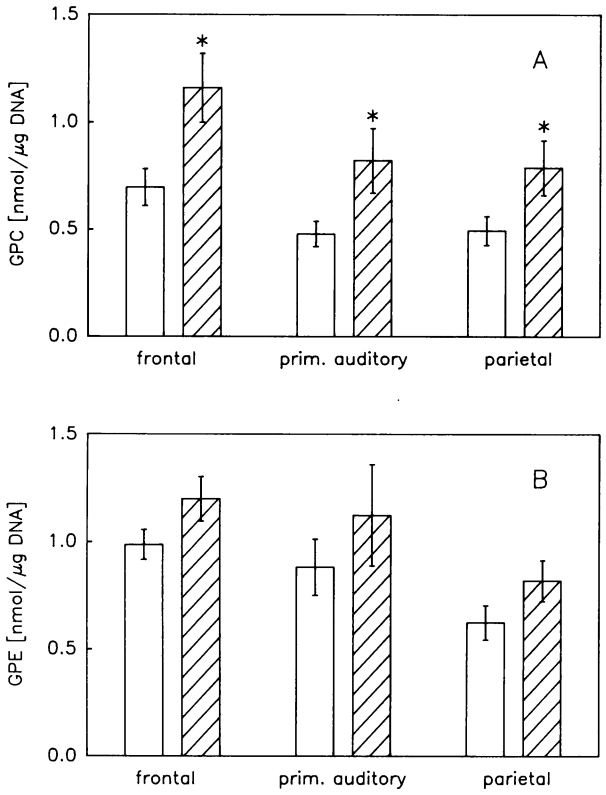

FIG. 3. Levels of phospholipid catabolites in human brain cortex: GPC (*A*) and GPE (*B*) in control (open bars) and AD (hatched bars). *, $P < 0.05$ versus respective control (analysis of variance). $n = 10$ for each group and area.

Statistical Analysis. Data were expressed as mean ± SEM. To test the one-sided alternative hypothesis that levels of individual phospholipids are lower in AD brain, one-tailed *t* tests were applied. Statistical analyses of all other data were performed using analysis of variance and Scheffe *post hoc* tests for group comparisons. Statistical significance was assumed at $P < 0.05$.

RESULTS

Phospholipid Levels. PtdCho and PtdEtn were decreased by 12–15% in frontal and parietal cortex ($P < 0.05$) and unchanged in primary auditory cortex (Fig. 2). Phosphatidylserine, sphingomyelin, and the sum of phosphatidic acid plus phosphatidylinositol did not differ significantly in any of the examined groups (Table 2).

Phospholipid Catabolites. GPC levels were increased ($P < 0.05$) in all examined AD brain regions (Fig. 3*A*). GPE levels were not significantly different (Fig. 3*B*). GPC levels in frontal cortex of Down syndrome, Huntington disease, and Parkinson disease brains did not differ significantly from control values (Table 3); GPC levels in Huntington disease and Down syndrome brains were lower ($P < 0.05$) than in AD. GPC levels in Parkinson disease brains did not differ significantly from either control or AD group values.

Initial Phospholipid Precursors. Brain levels of choline were decreased by 40–50% ($P < 0.05$) in AD frontal and parietal cortex (Fig. 4*A*). Choline levels in AD did not differ significantly from the mean values in Huntington's disease, but they were significantly lower ($P < 0.05$) than mean values for Down syndrome and Parkinson disease brains (Table 3). Brain ethanolamine levels were decreased by 33–50% ($P < 0.05$) in AD compared with control brains (Fig. 4*B*). Cortical levels of serine, a precursor for phosphatidylserine, were normal in AD (Fig. 4*C*).

Product-to-Precursor Ratios. In frontal and parietal cortex, the GPC-to-choline ratio was 3-fold higher ($P < 0.01$) in AD than in control brain (Fig. 5*A*); in primary auditory cortex this ratio was 2-fold higher ($P < 0.05$). The GPE-to-ethanolamine ratio was consistently 2-fold higher in AD in all examined brain regions ($P < 0.01$ for frontal and parietal cortex; $P < 0.05$ for primary auditory cortex) (Fig. 5*B*).

Phosphomonoesters. Levels of phosphocholine and phosphoethanolamine were normal in AD brain (Table 4). Phosphocholine levels were significantly higher in AD than in Huntington disease, but they did not differ from those in Down syndrome and Parkinson disease (Table 3).

GPC Cholinephosphodiesterase Activity. In homogenates of human parietal cortex, [^{14}C]GPC was hydrolyzed to phospho[^{14}C]choline, indicating GPC cholinephosphodiesterase activity. This activity was 268 ± 71 nmol/mg of protein per hr in controls ($n = 5$), and 252 ± 54 nmol/mg of protein per hr in AD brains ($n = 5$). There was no significant difference between the two groups. There was no significant formation of [^{14}C]choline in any of the examined human tissue homogenates.

DISCUSSION

The results of this study show that phospholipid metabolism is abnormal in AD brain cortex as indicated by a depletion of phospholipids in AD brain: tissue levels of the two major phospholipid classes, PtdCho and PtdEtn, were 10–12% lower in parietal and frontal cortex of AD patients than in brains of control subjects. This decrease represents an average phospholipid loss of 2 nmol/μg of total tissue DNA. Given that AD is characterized by neuronal rather than glial degeneration, the phospholipid depletion of neurons as calculated on a percent basis is probably underestimated by measurements of total tissue homogenates. A depletion of phospholipid molecules of the magnitude described here is compatible with accelerated membrane degeneration in AD brain.

Additionally, we found significant elevations in cortical levels of the PtdCho catabolite GPC. GPC is generated by deacylation of PtdCho (17). It is therefore likely that the accumulation of GPC in AD brain reflects increased activity of the deacylation pathways mediated by phospholipase A and lysophospholipase (see Fig. 1). Increased levels of GPC

Table 3. Levels of choline-containing phospholipid metabolites in human frontal cortex in various neurodegenerative diseases

Diagnosis	*n*	Metabolite, nmol/μg of DNA		
		GPC	Phosphocholine	Choline
Huntington disease	5	0.729 ± 0.207	0.239 ± 0.096*†	0.105 ± 0.097*‡
Parkinson disease	3	0.879 ± 0.088	0.379 ± 0.070	0.312 ± 0.110
Down syndrome	5	0.603 ± 0.085	0.382 ± 0.049	0.204 ± 0.020
Control	10	0.696 ± 0.086	0.521 ± 0.074	0.281 ± 0.046
AD	10	1.160 ± 0.160*§	0.494 ± 0.066	0.139 ± 0.018*‡

*, $P < 0.05$ vs. control; †, $P < 0.05$ vs. Parkinson disease, Down syndrome, and AD; ‡, $P < 0.05$ vs. Parkinson disease and Down syndrome; §, $P < 0.05$ vs. Huntington disease and Down syndrome.

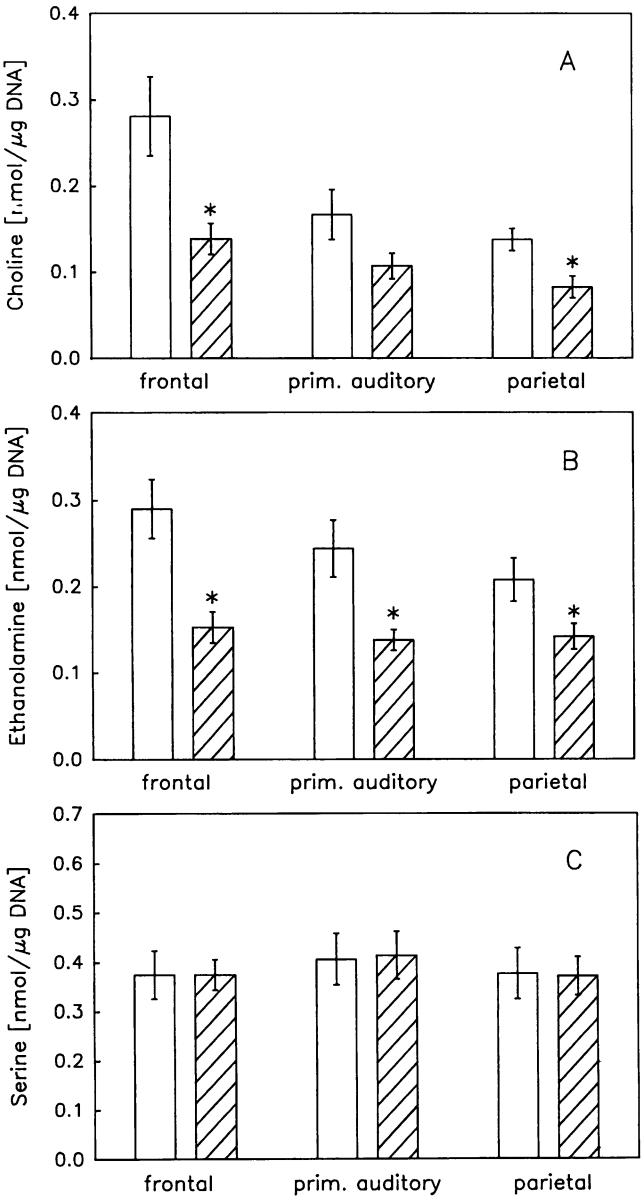

FIG. 4. Levels of the initial phospholipid precursors in human brain cortex: Choline (*A*), ethanolamine (*B*), and serine (*C*) in controls (open bars) and AD (hatched bars). *, $P < 0.05$ versus respective control (analysis of variance). $n = 10$ for each group and area.

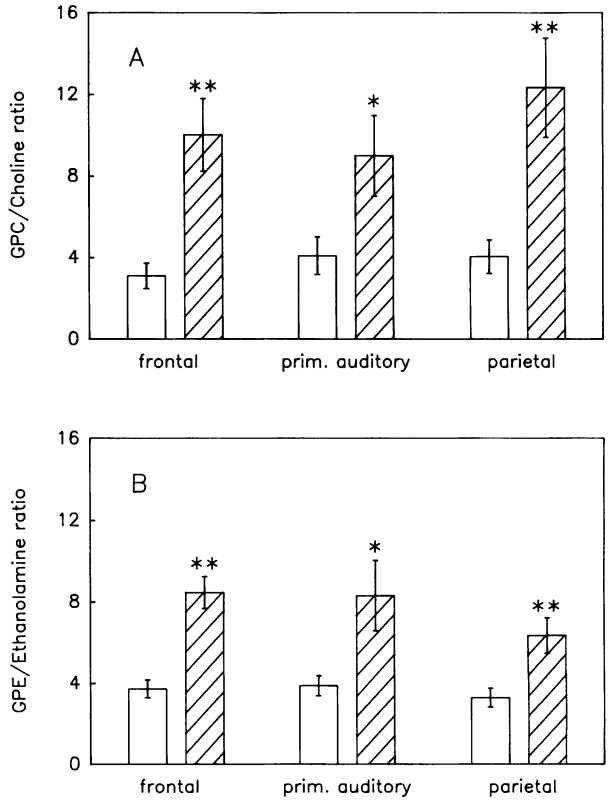

FIG. 5. Ratios of phosphodiester to free phospholipid base in human brain cortex: GPC-to-choline ratio (*A*) and GPE-to-ethanolamine (*B*) ratio in controls (open bars) and AD (hatched bars). Ratios were calculated as indicated in *Experimental Procedures*. *, $P < 0.05$; **, $P < 0.01$ vs. respective control (analysis of variance). $n = 10$ for each area and group.

cannot be attributed to impaired GPC degradation because GPC cholinephosphodiesterase activity was normal. The sum of the increase of GPC and GPE in AD brain was in the range of 1–1.5 nmol/μg of DNA, suggesting close stoichiometry between increased phospholipid catabolism and GPC accumulation.

GPC levels in brains of five Down syndrome patients were normal, confirming our previous report (8). Down syndrome is similar to AD in many pathoanatomical (18) and biochemical measures (19). In fact, the Down syndrome patients studied here had more cortical destruction and higher amounts of senile plaques and neurofibrillary tangles than the AD patients. GPC levels in Parkinson disease and Huntington disease brains were normal, suggesting that the elevation of GPC may be a distinctive characteristic of AD. Available data do not allow us to determine if the changes in levels of phospholipids and their catabolites reflect a primary defect in membrane metabolism leading to AD. However, the normal GPC levels in Down syndrome, Parkinson disease, and Huntington disease indicate that amyloid deposition or neurodegeneration *per se* are not necessarily accompanied by GPC accumulation.

The major pathways for PtdCho and PtdEtn synthesis involve phosphorylation of free choline and ethanolamine by kinases to form the corresponding phosphorylated derivatives phosphocholine and phosphoethanolamine (20). Choline and ethanolamine—as well as phosphocholine and phosphoethanolamine—are also formed by phospholipid catabolism and may therefore be considered as both precursors and degradation products of PtdCho and PtdEtn, respectively (see Fig. 1). We measured all four compounds and found decreased amounts of choline and ethanolamine in AD brain but unchanged levels of phosphocholine and phosphoethanolamine. These observations confirm the previous report of a 30–50% reduction in ethanolamine in AD brain cortex (21);

Table 4. Levels of phosphomonoesters in human postmortem brain cortex

Area	Group	*n*	Phosphomonoester, nmol/μg of DNA: Phosphocholine	Phosphomonoester, nmol/μg of DNA: Phosphoethanolamine
Frontal	Control	10	0.521 ± 0.074	0.395 ± 0.029
	AD	10	0.494 ± 0.066	0.347 ± 0.045
Prim. aud.	Control	10	0.426 ± 0.063	0.332 ± 0.040
	AD	10	0.443 ± 0.046	0.264 ± 0.038
Parietal	Control	10	0.357 ± 0.055	0.278 ± 0.027
	AD	10	0.300 ± 0.060	0.251 ± 0.021

Prim. aud., primary auditory cortex. No statistically significant differences were found between the AD and control groups.

there are no prior data on choline levels in AD brain tissue. The estimation of brain choline and ethanolamine levels is complicated by the fact that these compounds accumulate markedly during the initial postmortem time period (22, 23). In contrast, GPC levels appear to be relatively stable during this period (24). To minimize variations arising from postmortem time interval artefacts, the tissue samples investigated in this study were pairwise matched on the basis of time interval between death and autopsy.

Our data are consistent with increased utilization of choline and ethanolamine and increased phospholipid turnover, as indicated by the 2- to 3-fold increase in GPC-to-choline and GPE-to-ethanolamine ratios. Decreased levels of choline and ethanolamine in AD brain could also imply reduced availability of the precursors for PtdCho and PtdEtn synthesis. Furthermore, a decrease in choline levels may slow acetylcholine synthesis because of the high K_m of choline acetyltransferase for choline as substrate (30–100 μM) (17), and thus contribute to the cholinergic deficit in AD.

Abnormal metabolism in AD brain did not affect all phospholipid classes: neither serine nor phosphatidylserine levels were changed in AD brain. PtdCho and PtdEtn are similar in their metabolic pathways, whereas phosphatidylserine differs from PtdCho and PtdEtn in the mode of formation, the distribution within membranes, the degradation rate, and the fatty acid composition (25).

Tissue levels of the phosphomonoesters phosphocholine and phosphoethanolamine were normal in AD brain. These data are consistent with the previous findings of unchanged phosphoethanolamine levels in AD parietal and occipital cortex (21), as well as in hippocampus and substantia nigra (26). In other brain regions, significant decreases in phosphoethanolamine have been described (21, 26). These prior reports and our current data contradict the reported increase of phosphomonoesters in AD brain measured by ^{31}P NMR spectroscopy (5). One explanation for the divergent results might be time dependence: phosphomonoester levels might be elevated only in the intermediate stage but become normal in the late and end stages of AD (3). Our data are not suitable for testing this suggestion because all of our AD patients had severe advanced dementia.

One consequence of abnormal phospholipid metabolism could be enhanced amyloid deposition. Amyloid formation in AD brain requires abnormal processing of the amyloid precursor protein (APP) (27, 28). Defective membrane metabolism could expose the APP transmembrane domain to proteolytic cleavage; alternatively, amyloidogenic APP fragments may be poorly anchored to defective membranes and thus released into the neuropil. In support of this concept, data from *in vitro* experiments suggested that self-aggregation of amyloidogenic APP fragments is inhibited in the presence of membranes (1). Mechanisms of amyloidogenesis in AD may differ from those in Down syndrome, in which phospholipid metabolism is normal. In Down syndrome, it has been proposed that amyloid deposition is due to the 1.5-fold increase in APP gene dosage (29). In AD, however, the APP gene dose is normal (30–32).

In conclusion, our results strongly support the contention that brain cell membrane degeneration is accelerated in AD: increased degradation of the membrane phospholipids PtdCho and PtdEtn was paralleled by the depletion of free choline and ethanolamine and accumulation of GPC in brain tissue. Consequences of abnormal membrane metabolism may include slowed synthesis of phospholipids and acetylcholine. Because similar changes did not occur in brains of patients with Down syndrome, Huntington disease, or Parkinson disease, we postulate that the alterations of phospholipid metabolism in AD may reflect a distinct biochemical lesion related to the pathophysiology of AD.

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