Reduced Phosphatidylinositol Kinase Activity in Alzheimer’s Disease: Effects of Age and Onset

Key Words
Alzheimer
Dementia
Phospholipids
Phosphatidylinositol
Phosphatidylinositol kinase

Abstract
Phosphatidylinositol kinase (PI kinase) and phosphatidylinositol phosphate kinase (PIP kinase) were assayed in a membrane-free cytosolic fraction prepared from the medial temporal cortex of patients with Alzheimer’s disease (AD) and nondemented controls, with exogenous lipids as substrate. $^{32}$P-PIP formation appeared to be reduced by 21% in patients with AD who died before 80 years, compared to matched controls. In addition, there was an age-related decrease in PI kinase activity in the control group. In the AD patients there was no age-related decrease. Very old AD patients (older than 80 when they died) with a short duration of the disease were characterized by increased PI kinase activity. No differences were found in the closely related enzyme PIP kinase. The results are indicative for heterogeneity in AD.

Introduction

Alzheimer’s disease (AD) is a neurodegenerative disease and the most common cause of dementia. The etiology and pathogenesis are presently not known. Interest in brain membrane phospholipids in AD was aroused several years ago when it was found that cholinergic neurons especially are vulnerable to the degenerative process of Alzheimer’s disease [e.g., ref. 1]. These neurons utilize choline both for the synthesis of acetylcholine and for the formation of the membrane-phospholipid phosphatidylcholine. Alterations in PC and its metabolites, and in other phospholipids in AD brain have now been described [2–4].

Recently, cytosolic fractions obtained from neocortical structures taken from brains of AD patients were found to have approximately 50% less activity in phosphatidylinositol (PI) kinase activity than similar fractions obtained from control brains. No such differences were found in the activity of the closely related enzyme phosphatidylinositol 4-phosphate (PIP) kinase [5]. Stokes and Hawthorne [6] had earlier reported that the AD brain is characterized by a decreased content of PI. Phospholipids which have the sugar moeity inositol as their polar head group are particularly important for membrane functioning. Nervous tissue is especially rich in inositol phospholipids, which are involved in neurochemical processes related to neurotransmission [for review, see, ref. 7, 8]. Specifically, this class of compounds is implicated in age-
nist-receptor coupling, Ca$^{2+}$ gating and Ca$^{2+}$ mobilization from internal stores, and the formation of the intracellular second messengers diacylglycerol and inositoltrisphosphate [9].

The present study elaborates on the findings of an earlier study [5]. The focus is not on phosphoinositide levels but on the formation of PIP and phosphatidylinositol 4,5-bisphosphate (PIP$_2$). Phosphoinositides are very labile and are rapidly broken down after death [10, 11], which makes it difficult to study these substances in brain material obtained hours after death. It was therefore decided to investigate the activity of the enzymes involved in the interconversion of the inositol phospholipids and not the concentration of the inositol phospholipids. PI kinase and PIP kinase were studied in a membrane-free supernatant of medial temporal cortex prepared from AD and control brains, with exogenous PI and PIP as lipid substrate. Care was taken to use brain material from rapid autopsies (between 4 and 6 h after death). In the first experiment, we investigated the possible importance of the factor age: groups of AD patients and controls younger and older than 80 years at death were compared. This age was chosen in view of the finding of Rossor et al. [12] that there are differences in the neurotransmitter systems of AD patients who were younger than 79 years when they died compared to patients who were older when they died. In a second experiment, we investigated the age of disease onset in patients older than 80 years, in view of increasing clinical evidence that the clinical course of AD is heterogeneous. Very old patients who develop the first symptoms at great age (e.g., above 75 years) are often characterized by depression and psychosocial changes. In this study we set the age of onset of first symptoms arbitrarily at 75 years and compared old AD patients with an onset before or after 75 years.

Table 1. Patient characteristics of study 1: effect of age

<table>
<thead>
<tr>
<th></th>
<th>Alzheimer aged</th>
<th>Alzheimer very old</th>
<th>Control aged</th>
<th>Control very old</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>8</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Age mean, years</td>
<td>70.4 ± 2.4</td>
<td>83.2 ± 0.8</td>
<td>68.1 ± 2.2</td>
<td>86.0 ± 1.4</td>
</tr>
<tr>
<td>Age range, years</td>
<td>55–79</td>
<td>81–88</td>
<td>51–76</td>
<td>82–90</td>
</tr>
<tr>
<td>Male/female</td>
<td>7 M/3 F</td>
<td>0 M/8 F</td>
<td>8 M/3 F</td>
<td>3 M/3 F</td>
</tr>
<tr>
<td>Duration of illness, years</td>
<td>10.4 ± 1.9</td>
<td>14.4 ± 3.2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Brain weight, g</td>
<td>1,101 ± 38</td>
<td>1,010 ± 25</td>
<td>1,314 ± 34</td>
<td>1,188 ± 48</td>
</tr>
<tr>
<td>Post mortem delay, min</td>
<td>233 ± 16</td>
<td>231 ± 7</td>
<td>384 ± 93</td>
<td>348 ± 43</td>
</tr>
<tr>
<td>pH of the brain</td>
<td>6.6 ± 0.2</td>
<td>6.6 ± 0.1</td>
<td>6.5 ± 0.1</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>Choline acetyltransferase</td>
<td>0.48 ± 0.16</td>
<td>0.46 ± 0.21</td>
<td>5.77 ± 0.77</td>
<td>3.68 ± 0.47</td>
</tr>
</tbody>
</table>

Table 2. Patient characteristics of study 2: effect of onset

<table>
<thead>
<tr>
<th></th>
<th>Alzheimer onset = 75</th>
<th>Alzheimer onset &gt; 75</th>
<th>Control onset = 75</th>
<th>Control onset &gt; 75</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Age mean, years</td>
<td>83.3 ± 0.8</td>
<td>88.3 ± 0.7</td>
<td>86.0 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Age range, years</td>
<td>81–88</td>
<td>85–90</td>
<td>82–90</td>
<td></td>
</tr>
<tr>
<td>Mean age of onset</td>
<td>68.9 ± 2.9</td>
<td>80 ± 1.0</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>0 M/8 F</td>
<td>0 M/7 F</td>
<td>3 M/3 F</td>
<td></td>
</tr>
<tr>
<td>Duration of illness, years</td>
<td>14.4 ± 3.2</td>
<td>8.3 ± 1.1</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Brain weight, g</td>
<td>1,010 ± 26</td>
<td>961 ± 27</td>
<td>1,188 ± 48</td>
<td></td>
</tr>
<tr>
<td>Post mortem delay, min</td>
<td>231 ± 7</td>
<td>221 ± 16</td>
<td>347 ± 43</td>
<td></td>
</tr>
<tr>
<td>pH of the brain</td>
<td>6.6 ± 0.1</td>
<td>6.4 ± 0.1</td>
<td>6.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Choline acetyltransferase</td>
<td>0.46 ± 0.21</td>
<td>1.01 ± 0.32</td>
<td>3.68 ± 0.47</td>
<td></td>
</tr>
</tbody>
</table>

Materials and Methods

Subjects

Brain samples from patients with AD and nondemented controls were obtained from the Netherlands Brain Bank in Amsterdam. For the first experiment, 18 AD brains and 17 controls were used. Subgroups were composed of 'aged' subjects, aged 80 or younger, and 'very old' subjects, older than 80 years. Care was taken to include only patients in whom AD started when they were 75 or younger. The duration of illness did not differ between aged and very old AD subjects (see table 1 for description). In all cases the interval between death and autopsy did not exceed 5 1/4 h: the mean postmortem delay was 240 and 230 min for the aged and very old AD groups and 320 and 310 for the controls, respectively. The difference in postmortem delay was not significant. In the second experiment, 15 AD patients aged 81–90 years were divided into two subgroups which differed with respect to age of onset (75 years and younger, or over 75) and compared with 6 control subjects aged 82–90 years. There was a statistically significant difference in the mean duration of illness between the two AD groups (14.3 ± 3.2 versus 8.3 ± 1.1; see table 2 for description). The patients with AD were clinically diagnosed as 'probable AD' [13, 14], and this was verified by postmortem
neuropathologic examination. Control subjects had no history of dementia or any other neurological or psychiatric disorder.

**Brain Dissection**

Brain specimens for analysis of inositol phospholipid kinase activity were taken from the medial temporal gyrus in the right hemisphere. The leptomeninges were removed and samples were excised measuring approximately 1 cm³. These samples were sealed in plastic and rapidly frozen by immersion in liquid nitrogen. The frozen samples were stored at -80 °C until use.

**Preparation of Crude Enzyme Fraction**

Pieces of approximately 0.5 g were excised from the tissue samples and thawed in a water bath at 0 °C (20 min). The tissue was homogenized in medium consisting of 0.32 M sucrose, 1 mM EGTA, and 50 mM Tris-HCl, (pH 7.4) in a total volume 10 times the brain tissue volume, by 12 up-and-down strokes (16 s each) of a Potter-Elvehjem Teflon glass homogenizer (radial clearance, 0.125 mm; 700 rpm), followed by homogenization by hand in a glass-glass homogenizer with 3 up-and-down strokes (clearance of cylindrical section: 0.125 mm) in order to destroy synaptosomes and thus increase the yield of intra-synaptosomal enzymes. The homogenate was centrifuged for 60 min at 100,000 g and the resulting membrane-free supernatant was used as the crude enzyme fraction. This fraction was frozen in 0.2-ml aliquots in liquid nitrogen for 5 s and stored at -80 °C. There was no decline in enzyme activity after 1 month of storage.

**PI Kinase and PIP Kinase Assay**

Inositol phospholipid kinase activity was measured, with some modifications, as described by Van Dongen et al. [15] and Moritz et al. [16]. The incubation volume (normally 25 μl) was doubled in the case of the PIP kinase assay in order to reduce interassay variability. Supernatant fractions of 15 or 30 μl (10 and 20 μg of protein, respectively) were preincubated for 2 min. Lipid precursors (20 μM PI or 20 μM PIP; Sigma) were solubilized in 0.1% Triton X-100, 50 μM Tris-HCl, and 1 mM EGTA (pH 7.4) and added to the incubation mixture 15 s before the phosphorylation reaction was started by addition of ATP. The reaction lasted 1 min. Incubations were performed under the following conditions: 7.5 μM ATP, 2-3 μCi [32P]ATP (approximately 3,000 Ci/mmol, Amersham, UK), 50 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 1 mM EGTA, and 0.02% Triton X-100. The reaction was terminated and the extraction and further analysis of the 32P incorporated into PIP and PIP₂ were performed as described elsewhere [17, 18]. Protein content was determined according to the method of Lowry et al. [19].

**Results**

In the first experiment, a comparison was made between aged AD patients (who were younger than 80 when they died) and very old AD patients (who were older than 80 when they died), in whom the onset of disease occurred before 75 years, and age-matched controls. The pH of the brain, as a measure of post-mortem delay, did not differ between any of the groups. However, the groups were different with respect to the factor brain weight (see table 1). The brains of AD patients weighed less than those of controls (p < 0.001; Student-Newman-Keuls test). In addition, there was an effect of the factor 'age': the brains of AD patients and control subjects older than 80 weighed less than the brains of subjects younger than 80 (p < 0.01). Remarkably, the mean brain weight of very old controls (1,188 g) was not different from that of aged AD patients (1,102 g). Choline acetyltransferase activity was reduced by 90% in both aged and very old AD brains. Interestingly, the older controls were characterized by a significant decrease in choline acetyltransferase activity compared to younger controls.

Data on PI kinase activity were analyzed with a two-way ANOVA with factors group and age. A group effect was found and an interaction age × group. Thus, as shown in figure 1, there was a reduction in PI kinase activity in aged AD patients aged <80 years, compared to matched controls (p < 0.05). This group effect was absent in very old subjects aged >80 years because of the reduction in PI kinase activity in very old controls compared to controls aged <80 years. A regression analysis of PI kinase activity versus age (fig. 2) indicated that PI kinase activity decreased with age (p < 0.05). Furthermore, in agreement with findings presented elsewhere [5], AD patients and controls had similar PIP kinase activity as manifested by the formation of PIP₂ (results not shown).
In addition, age did not affect PIP kinase activity – PIP₂ formation – in either AD patients or controls.

The second experiment was devised to analyze the possible influence of the factor 'onset'. Very old AD patients and controls older than 80 were compared. As table 3 shows, PI kinase activity in AD patients in whom the onset of first symptoms occurred before and after 75 years were significantly different. Very old AD patients in whom AD started when they were older than 75 had increased PI kinase activity compared to AD patients with an earlier onset and controls. Remarkably, the PI kinase activity in this group was even higher than in the younger controls measured in experiment 1 (20% increase, p < 0.01). The incorporation of ³²P into PIP₂ was similar in patients in whom the onset of AD occurred after 75 years and in patients with an earlier onset (results not shown).

**Table 3. PI kinase activity in old patients: effect of onset**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>PI kinase activity pmoi/min·mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onset ≤ 75 years</td>
<td>8</td>
<td>3.719 ± 0.200</td>
</tr>
<tr>
<td>Onset &gt; 75 years</td>
<td>7</td>
<td>5.523 ± 0.484</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>3.125 ± 0.314</td>
</tr>
</tbody>
</table>

**Discussion**

Recently, cytosolic PI kinase fractions obtained from neocortical structures taken from brains of patients with AD were found to have approximately 50% less activity than similar fractions obtained from control brains. No such differences were detected in PIP kinase activity [5]. The fractions were prepared from patients with young-onset AD who died at a relatively early age (below 72 years). The present series of investigations confirms this finding for a larger group of subjects, some of whom lived to be 80. Interestingly, very old controls (older than 80, with a mean age of 86 years) had a reduced PI kinase activity compared to younger controls (younger than 80, with a mean age of 68.1 years), and evidence for an age gradient in PI kinase activity was found. The decreased activity in old controls could explain the lack of difference between old controls and very old AD patients (older than 80) with onset before 75 years in the present series of investigations. The factor 'onset' – which is defined as 'age at first symptoms' – was found to be important in the second experiment in which old AD subjects with a relatively long duration of disease (mean duration 14.4 years, onset before 75) were compared with AD patients with a short duration of disease (mean duration 8.3 years; onset after 75). The results clearly show that the latter group is characterized by high PI kinase activity.

The present findings are important for both methodological and theoretical considerations. Age-related decreases in myo-inositol and inositol bound to phosphatiidylinositol have been found in normal subjects aged 20-70 [20]. An age-related decrease in PI kinase activity, found in the present study, has relevance for AD research, as the lack of brain material from well-defined AD patients forces the researcher to use groups which are composed of AD patients of widely different ages [e.g., 60 through 90 years; ref. 6]. The present data suggest that care should be taken in the selection of patient groups. The use of control and patient groups that are more homogenous with respect to age is to be recommended in view of the age-related decline in enzyme activity.

Old AD subjects with an onset before and after 75 years had different PI kinase activity, which could be important in view of the fact that the late-onset group showed an increased formation of ³²P-PIP compared to the early-onset group, whereas the late onset group was older and thus – in view of the findings of the first study – would be expected to have a decreased formation of PIP. In addition, old AD patients with a short disease duration (onset after 75 years) had a much higher PI kinase activity.
than subjects with a longer disease duration. In conclusion, the present data suggest that the pathogenesis of AD in old AD patients with a short duration of disease might be different, as far as PI kinase is concerned, from that of old patients with a longer duration of disease. This is of relevance in view of the ongoing discussion on the possible heterogeneity of AD.

The present data do not enable a conclusion as to whether ‘age of onset’ is the relevant variable or ‘duration of disease’. However, it is most probable that age of onset is the more important, in view of our finding that the younger AD patients with short disease duration are especially reduced in PI kinase, compared to matched controls [this paper and ref. 5]. Also, other papers refer to the importance of the factor ‘onset’ [e.g. ref. 21].

Until now, the question whether or not early- and late-onset AD differ has not been settled. An increasing number of studies favor such a distinction in order to account for clinical heterogeneity and different syndromes which are observed in early- and late-onset AD [21–23]. Likewise, there is increasing evidence that early- and late-onset AD also differ with respect to neurological parameters [24, 25]. Even age at death appears to be a relevant variable in the differentiation of AD patients in view of the findings reported by Rossor et al. [12]: these authors found clearcut differences in neurotransmitter systems in AD patients younger than 79 years versus AD patients dying at an older age. The present findings corroborate these observations for another enzyme system. In addition to age at death, age at onset of first symptoms also appeared to be relevant, which suggests that it might be necessary to reevaluate existing data in order to find whether our observations on the effect of onset are paralleled by similar early/late discrepancies found in other neurological or neurochemical parameters.

Both the present results and those presented by Stokes and Hawthorne [6] and Stokes et al. [20] suggest that the inositol phospholipid system may be involved in the pathogenesis of AD. Stokes and Hawthorne [6] measured absolute levels of myo-inositol and lipid-bound inositol, and found a significant decrease in PI in the temporal cortex from AD brain. In addition, an age gradient for myo-inositol and lipid-bound inositol was found, although there was a large individual variation. Several other investigators have reported the involvement of membrane phospholipids and their metabolites in Alzheimer’s disease, for instance, by 31P-NMR spectroscopic examination of AD brains. Elevated levels of phosphomonoesters such as phosphocholine early in the course of the disease have been reported, followed by elevation of phospho-
diesters such as glycerol-3-phosphocholine [2–4, 26, 27]. In addition, AD brain appears to be characterized by a decrease in phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and cholesterol [4]. It remains to be established whether there is a relation between these changes and the findings reported in the present paper.

The changes in PI kinase activity seem to be relevant because of the large effect found, especially in young AD patients [5] and because of the key role played by inositol phospholipids in impulse initiation and propagation, the most intrinsic neuronal and brain functions [see ref. 7, 8 for reviews]. A large decrease in PIP formation can therefore be expected to have a widespread influence on membrane function. PIP2 hydrolysis could be blocked, and the formation of the second messengers inositol phosphate and diacylglycerol inhibited. This in turn can be expected to give rise to a number of tertiary changes in intracellular mechanisms and lead to neuronal deactivation and possibly degeneration.

With respect to the biochemical mechanisms involved in the reduced incorporation of 32P into PIP with age and in AD patients, changes in the absolute amount of enzymes or changes in enzyme kinetics are possible. Alternatively, the presence or absence of particular ions or other cofactors could also be causal to the decreased incorporation. Follow-up research into the various possibilities is presently being undertaken. In addition, uncertainty exists about the nature of the PI kinase involved. It is known that this enzyme exists in two forms, namely the kinase which phosphorylates PI at the 4 position of the inositol ring, and the one which phosphorylates at the 3 position. This difference is of importance because the two kinases appear to have different functions in the cell. The PI-4-kinase is involved in the pathways leading to hormone-stimulated phospholipase C action, whereas the PI-3-kinase is suggested to have a function in mitogenesis, cell differentiation, and in the control of cytoskeletal rearrangements [9, 28]. Various isoenzymes of PI-4-kinase exist, which are located either in the membrane as an integral membrane protein [e.g. ref. 29], or in the cytoplasm, or both [28].

In conclusion, the alterations in inositol phospholipid phosphorylation found in the present study may be relevant for AD research because of the key role played by inositol phospholipids in neural functioning. In addition, the results indicating an age-related effect in AD patients suggest that PI kinase activity can be used to divide AD patients into subgroups characterized by the age of onset of the disease. More attention should be given to the factors age and onset in future AD research.
References


Jolles/Bothner/Markerink/Ravid

PI Kinase in Alzheimer's Disease