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Platelet phospholipase A_2 activity in Alzheimer's disease and mild cognitive impairment

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Summary. Phospholipase A_2 (PLA_2) controls the metabolism of phospholipids in cell membranes. In the brain, PLA_2 influences the processing of the amyloid precursor protein (APP) and thus the production of the amyloid-beta peptides (Aβ), which are the major components of the senile plaques in Alzheimer's disease (AD). Reduced PLA_2 activity has been reported in brain and in platelets of AD patients. In the present study we investigated PLA_2 activity in platelets from 21 AD patients as compared to 17 healthy elderly controls and 11 individuals with mild cognitive impairment (MCI). Subjects were cognitively assessed by the Mini-Mental State Examination (MMSE) and the CAMDEX schedule. Platelet PLA_2 activity was determined by radio-enzymatic assay, which mainly detected a calcium-independent form of the enzyme present also in the brain ($iPLA_2$). PLA_2 activity was significantly lower in AD than in controls ($p < 0.001$). Mean PLA_2 activity in MCI individuals was between the values of AD patients and controls, with a subgroup showing PLA as low as the lowest AD patients, but the differences from MCI were not significant from AD and control groups. Lower PLA_2 activity was significantly correlated with a worse cognitive performance both at the MMSE ($p = 0.001$) and the cognitive sub-scale of the CAMDEX inventory ($p = 0.002$). Our data replicate previous findings of reduced platelet PLA_2 activity in AD. Both reduced PLA_2 activity and the correlation with impaired cognition were also reported in brain tissue of AD patients, suggesting thus that the present determinations in platelets may be related to a reduction in the brain. In the brain the inhibition of PLA_2 inhibits the physiological secretion of the APP, a mechanism that increases Aβ formation. Further longitudinal studies should investigate whether those MCI individuals with the lowest PLA_2 values in platelets would be at a higher risk to develop AD during a longitudinal follow up.

Keywords: Dementia, Alzheimer's disease, mild cognitive impairment, brain phospholipids, phospholipase A_2, PLA_2, platelets.

Introduction

Phospholipases A_2 (PLA_2) are a super-family of enzymes that participate in a wide variety of physiological processes, including phospholipid metabolism, remodeling of cell membranes, and intracellular signalling. In most cell types, PLA_2 contributes to the release of arachidonic acid from membrane phospholipids, which is a fundamental step in the synthesis of major mediators of the inflammatory response. In cholinergic neurons, particularly, the cleavage of membrane phosphatidylcholine by PLA_2 has an additional role in release of choline, the main precursor for the synthesis acetylcholine (Blusztajn et al., 1987). PLA_{2s} have a preferential affinity for the cleavage of arachidonyl tails at the *sn*-2 position of phospholipid molecules (Clark et al., 1991). Abnormal regulation of highly unsaturated fatty acid metabolism by PLA_2 may result in modifications of the neuronal membrane structure and fluidity, and affect intracellular signalling and neurotransmitter- and ion channel-related functions (review in Horrobin, 1998).

Data suggesting increased PLA_2 activity in schizophrenia (Gattaz et al., 1987, 1990, 1995; Noponen et al., 1993; Ross et al., 1997) have raised the attention to the role of membrane abnormalities in neuropsychiatric disorders. Disordered PLA_2 metabolism has also been described in multiple sclerosis (Woelk et al., 1974), temporal-lobe epilepsy (Simonato, 1993; Visioli et al., 1994) and dyslexia (MacDonell et al., 2000).

In Alzheimer's disease (AD) there have been reports of decreased brain and platelet PLA_2 activity (Gattaz et al., 1995b, 1996). Within AD brains, reduced activity of the enzyme in the frontal and parietal cortex was related to earlier onset of dementia, earlier age at death, and higher counts of senile plaques and neurofibrillary tangles (Gattaz et al., 1996). In addition, ^{31}P-spectroscopy studies of AD patients have suggested decreased membrane phospholipid turnover in temporoparietal areas (Brown et al., 1989), which is compatible with the findings of decreased PLA_2 metabolites in the parietal cortex of AD brains (Skinner et al., 1989). At last, *in vitro* studies have implicated PLA_2 in the regulation of amyloid precursor protein (APP) processing in cell cultures (Emmerling et al., 1993). Taken together, evidence drawn from experimental and clinical studies support the hypothesis that abnormalities in membrane phospholipid metabolism, secondary to reduced PLA_2 activity, might play a role in the pathogenesis of AD.

In the present study we investigated PLA_2 activity in platelets of a larger sample of AD patients as compared to healthy elderly controls, and extended the investigation to a subgroup of non-demented individuals with mild cognitive impairment (MCI). Platelets are frequently used as peripheral markers for neurones because they share some common membrane and receptor properties, providing thus an interesting model for the investigation of metabolic abnormalities in Alzheimer's disease (Zubenko et al., 1999). In a second step, we attempted to characterise the subtype of PLA_2 detected by our assay in platelets.

Methods

Patients and controls

Twenty-one patients with probable ($n = 17$) or possible ($n = 4$) AD according to NINCDS-ADRDA criteria (McKhann et al., 1984), 11 subjects with evidence of mild cognitive

impairment (MCI), and 17 age-matched healthy controls were enrolled to the study. Written informed consent was obtained after the procedures had been fully explained. Alzheimer patients were sub-classified into early- and late-onset cases according to ICD-10 diagnostic criteria (World Health Organisation, 1992). The individuals with MCI were diagnosed according to modified ICD-10 criteria (e.g., complaints of cognitive function decline for at least 6 months, without any other psychiatric diagnosis or significant functional impairment). Cognitive status in all subjects was assessed by the Mini-Mental State Examination (MMSE) (Folstein et al., 1975) and the cognitive sub-scale (CAMCOG) of the Cambridge Mental Disorders of the Elderly Examination (CAMDEX) schedule (Roth et al., 1986), Portuguese version (Bottino et al., 1999). Severity of cognitive decline was assessed by the Clinical Dementia Rating scale (CDR) (Berg, 1984), being MCI patients' record by definition not greater than 0.5. Behavioural abnormalities and activities of the daily living were further evaluated by the Blessed inventory (Blessed et al., 1968).

Eleven AD patients were on drug treatment: Five patients received the AChE inhibitor rivastigmine alone ($n = 2$) or in combination with a neuroleptic drug ($n = 3$), 4 received a neuroleptic alone, one received imipramine and one received diazepam.

Data analysis was performed with the aid of the Statistical Package for the Social Sciences (SPSS/PC 10.0 for Windows). Likelihood ratio analysis of contingency tables using the Pearson's method was used in the investigation of categorical data, the statistical result being distributed as chi-squared (χ^2). Analysis of variance (ANOVA), with Bonferroni correction for post-hoc multiple comparisons, was used to compare group means. Student's t-test (t) was applied to compare the scores of outcome measures of normally distributed data. Parametric and non-parametric correlations (Pearson (r) and Spearman (rho) correlation coefficients) were obtained for normal or skewed/small sub-sample data.

Demographic data of cases and controls are presented in Table 1. There was a clear predominance of women (78%) in the sample as a whole, which was maintained in the three different sub-samples (71%, 91% and 76% respectively for female AD, MCI and control subjects, NS). There were no significant differences regarding the mean age, marital state, and years of schooling in the three groups.

Only one patient had a diagnosis of early onset familial AD, the other being classified as sporadic AD. Of these, 7 patients had disease onset before the age of 65 (sporadic early-onset) and 13 had disease onset after 65 (late-onset). Severe dementia occurred in 54% of late-onset and in 25% of early-onset cases. However, the disease's course length (months) and the severity of cognitive impairment (MMSE and CAMCOG) was not significantly different between late- and early-onset groups.

Table 1. Age, gender, marital status and education level in patients with Alzheimer's disease (AD), mild cognitive impairment (MCI), and healthy controls (means ± SD)

	AD ($n = 21$)	MCI ($n = 11$)	Control ($n = 17$)	Statistical test[1]
Age (years)	75.1 ± 6.9	72.8 ± 4.9	72.5 ± 4.6	$F = 1.11$ $p = 0.33$
Gender	6 M/15 F	1 M/10 F	4 M/13 F	$\chi^2 = 1.59$ $p = 0.45$
Married	33.3% ($n = 7$)	54.5% ($n = 6$)	42.9% ($n = 8$)	$\chi^2 = 1.51$ $p = 0.46$
Years of schooling	6.52 ± 4.34	7.45 ± 4.84	6.82 ± 3.74	$F = 0.17$ $p = 0.84$

M male; *F* female; [1] Age and Years of Schooling: ANOVA (F); Gender and Civil Status: Pearson Chi-Square (χ^2)

Table 2. Neuropsychological and clinical profiles of patients with Alzheimer's disease (AD), mild cognitive impairment (MCI), and healthy controls (means ± SD)

	AD (n = 21)	MCI (n = 11)	Control (n = 17)	ANOVA[2]
MMSE	14.29 ± 8.96*,**	24.82 ± 3.79	27.94 ± 2.07	F = 24.23 p < 0.001
CAMCOG Total score	44.14 ± 29.34*,**	76.27 ± 12.46	89.94 ± 6.01	F = 24.78 p < 0.001
CAMCOG Memory	7.43 ± 7.39*,**	16.64 ± 4.82***	22.65 ± 2.52	F = 35.84 p < 0.001
CAMCOG Attention	2.29 ± 2.51	4.91 ± 1.81	6.12 ± 1.11	F = 18.65 p < 0.001
Blessed Functional[1]	12.39 ± 5.05*,**	3.64 ± 2.65	2.18 ± 3.41	F = 20.4 p < 0.001
Blessed IMC	15.67 ± 11.59*,**	30.09 ± 4.01	34.12 ± 1.53	F = 28.08 p < 0.001

[1] n = 19, 7 and 8 respectively for AD, MCI and controls; [2] ANOVA with Bonferroni's post hoc test for multiple comparisons; * AD vs. MCI: $p < 0.001$; ** AD vs. controls: $p < 0.001$; *** MCI vs. controls: $p < 0.05$; *Blessed IMC* Blessed Information Memory and Concentration

The neuropsychological and clinical data are on Table 2. As expected, Alzheimer patients had a significantly worse performance both in memory and attention CAMCOG tasks as compared to MCI cases and controls. MCI cases had significantly worse performance than controls only on the CAMCOG memory subscale. Finally, AD overall cognitive scores were significantly lower than MCI and controls (Table 2).

Platelet isolation

Patients and controls had 40 ml of fresh blood collected in 0.1 M sodium citrate-coated tubes (S-Monovett, Sarsted). Blood samples were homogenised in 1 ml of acid citrate dextrose solution (ACD-NIH-formel-A solution: glucose 123.8 mM, tri-sodium citrate 83.9 mM, citric acid 41.3 mM) and centrifuged for 15 minutes at 515×G and room temperature (RT). Supernatants (platelet-rich plasma fractions) were pH adjusted to 6.5 and re-spun for 10 minutes at 1159×G (RT). Pellets were re-suspended in 5 ml of wash-solution (tri-sodium citrate 30 mM pH 6.5, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2 mM, NaCl 0.9%, glucose 5 mM, albumin 0.005%, apyrase 20 units/ml) and centrifuged for 8 minutes at 1159×G (RT). Platelet-rich pellets were then re-suspended in 500 µl of Tris-sucrose solution (Tris 50 mM pH7.4, sucrose 233 mM), counted, and stored at −70°C. Prior to radio-enzymatic assays, protein levels were determined for each aliquot by a modified Lowry method (Bio-Rad DC Protein Assay).

Determination of platelet PLA_2 activity

PLA_2 activity was determined in platelets by a radio-enzymatic assay. According to this method, ^{14}C-labelled arachidonic acid is preferentially cleaved by PLA_2 at the *sn*-2 position of the phosphatidylcholine molecule, and the radioactive product of the enzymatic reaction is measured in a liquid scintillation counter. An indirect estimate of PLA_2 activity (expressed in picomols per miligram of protein per minute) for each sample is ascertained by the total ^{14}C radioactivity count per minute, corrected by the respective protein levels and the reaction's coefficient of efficiency, which is determined for each count.

We used as substrate L-α-1-palmitoyl-2-arachidonyl-phosphatidyl-choline labelled with [l-^{14}C] in the arachidonyl tail at position *sn*-2 (^{14}C-PC) (New England Nuclear, Boston Massachussets), with specific activity of 48 mCi/mMol, previously diluted 1:10 (v/v) in a 0.14 g/ml

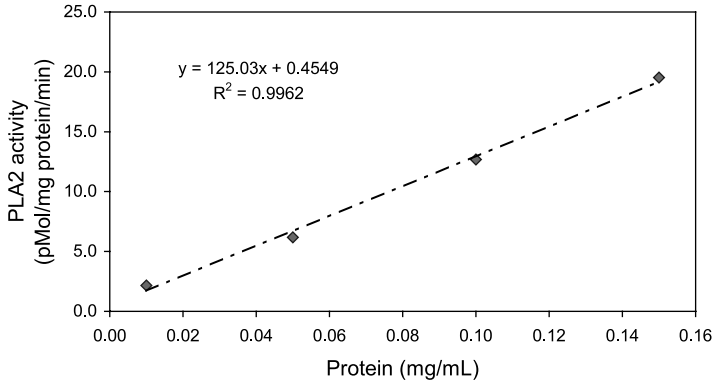

Fig. 1. Linear dependence of PLA_2 activity on protein concentration

solution of the antioxidant BHT (buthylated hydroxytoluene) in toluol-ethanol (1:2, v/v). Prior to enzymatic reaction, ^{14}C-PC solution was nitrogen evaporated in a glass vial (total volume equivalent to 0.06 mCi per aliquot = 12 µl per aliquot), re-suspended in 5 mg/ml human albumin solution (150 µl per aliquot), and homogenised by sonication. Platelet samples were diluted in 400 µl of Tris-sucrose solution to a concentration of 0.1 mg protein/ml, and the final volumes of the various aliquots were equalised to 1 ml by the addition of Tris buffer (50 mM pH 8.5). The aliquots for enzymatic reactions were prepared on ice with 200 µl of each diluted platelet sample (or respective blank controls), 100 µl of calcium chloride ($CaCl_2$ 25 mM), 50 µl of Tris (1 M pH 8.5), and 150 µl of the re-suspended radioactive ^{14}C-PC. Samples were then immersed in a 37°C shaking water-bath for 30 minutes, after which 700 µl of stop solution (hydrochloric acid-isopropanol 1:11.67 v/v) was added. Samples were allowed to rest for 10 minutes at room temperature in order to halt enzymatic reaction. Radioactive arachidonic acid (^{14}C-AA) released by the PLA_2 cleavage of ^{14}C-PC was then extracted by the *n*-heptane-silica method with Dole's reagent (Dole et al., 1960) followed by adsorption of phospholipids on silica (Sundaram et al., 1978). Samples were homogenised in 700 µl of *n*-heptane, and after a 5 minute, 3220×G, RT-spinning, 500 µl of the upper phase was transferred to silica-coated eppendorfs containing 300 µl of *n*-heptane. A second spinning was then performed for 10 minutes, after which 500 µl of the upper phase was added to the scintillation solution. Finally, the radioactivity of ^{14}C-AA was measured in a liquid scintillation counter (Packard Tri-Carb 2100 TR). All PLA_2 activity

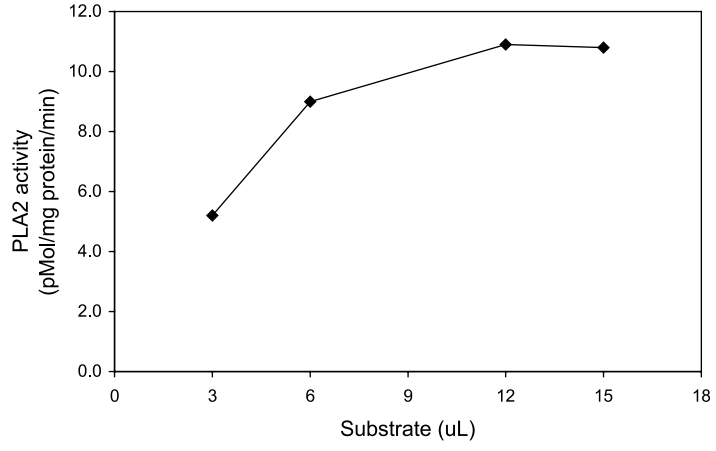

Fig. 2. Dependence of PLA_2 on substrate concentration

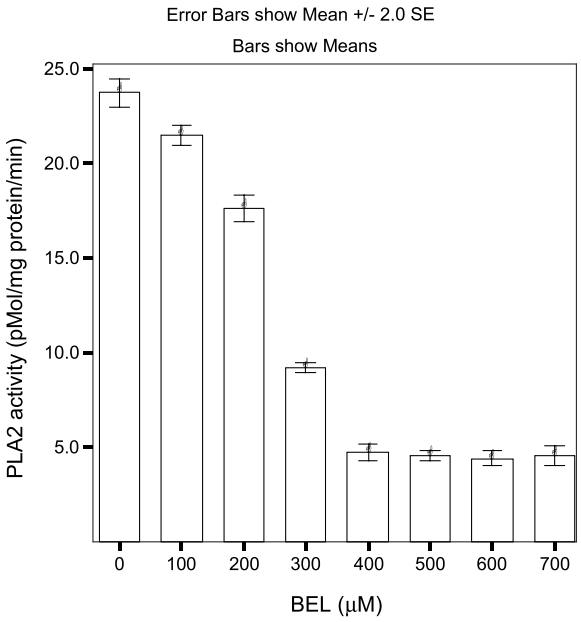

Fig. 3. Dose-curve of the inhibition of PLA_2 activity in platelets by bromoenol lactane (BEL). Maximum inhibition (73%) at 400 μM

determinations were performed in duplicates, and samples from AD, MCI and controls were run in the same assay. The enzyme activity is given in pMol/mg protein/min.

We evaluated the protein concentration and the substrate concentration at which PLA_2 activity is maximal in platelets. We found that the enzyme activity increases linearly with the protein concentrations in the range 0.01 to 0.15 mg/ml (Fig. 1). Moreover, the enzyme activity is maximal at the substrate concentration of 12 μl per aliquot containing 0.1 mg protein/ml (Fig. 2). Here all determinations were done in triplicate.

To evaluate the proportion of calcium-dependent PLA_2 and calcium independent PLA_2 ($iPLA_2$) detected by our assay, we determined the effect of bromoenol lactone (BEL, a suicide inhibitor for $iPLA_2$) on the total PLA_2 activity from human platelets. After the addition of calcium to the assay ($CaCl_2$ 5 mM, as described above), samples were treated with BEL in different concentrations, ranging from 100 to 700 μM. Results are in Fig. 3, in which each data point represents an average of three independent experiments, each in triplicate. There was a dose-dependent inhibition of PLA_2 by BEL, reaching the maximum inhibition (73% inhibition) by 400 μM BEL. Thus our assay detected in platelets both calcium-dependent and calcium-independent PLA_2 activities, accounting $iPLA_2$ for the major portion of the enzyme activity in platelets.

Results

Mean platelet PLA_2 activity was significantly different among the groups (ANOVA, $F = 9.27$, $p < 0.001$). Mean PLA_2 was lower in AD (18.8 ± 4.0) than in controls (28.3 ± 7.8) ($p < 0.001$). The enzyme activity in MCD patients (23.1 ± 6.6) was between the values in AD and controls, being these differences not significant from both groups (Fig. 4).

There were no differences in PLA_2 activity between AD patients with and without treatment with AchE inhibitors (18.5 ± 3.7 and 19.0 ± 4.3 respectively). AD patients on neuroleptic therapy showed lower PLA_2 activity than

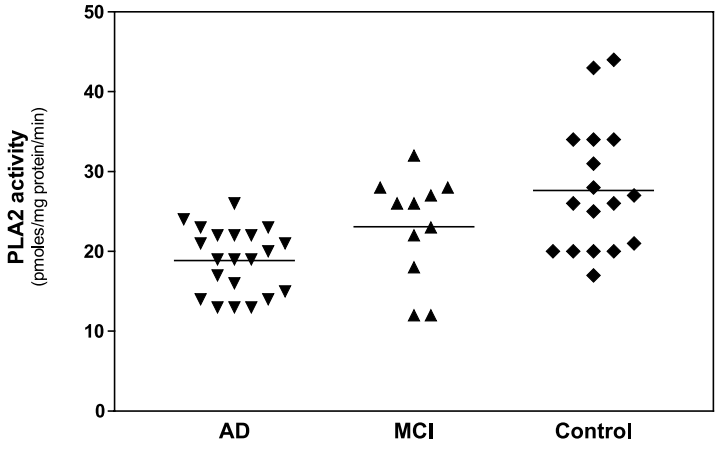

Fig. 4. Platelet PLA_2 activity in individuals with Alzheimer's disease (AD) ($n = 21$), mild cognitive impairment (MCI) ($n = 11$), and cognitively normal age-matched controls ($n = 17$) as determined by radio-enzymatic assays (pmol/mg protein/minute). Significance levels: AD vs. controls: $p < 0.001$

AD patients without neuroleptics (15.8 ± 3.0 and 20.3 ± 3.7, $p = 0.01$). However, excluding those 7 AD patients on neuroleptics from the analysis, the reduction of PLA_2 activity in AD patients remained significant as compared to controls (20.3 ± 3.7 vs 28.3 ± 7.8, $p = 0.01$).

In the whole sample (AD, MCI and controls together) PLA_2 activity correlated significantly with the degree of cognitive impairment, namely with MMSE scores ($r = 0.44$; $p = 0.001$), total CAMCOG ($r = 0.42$; $p = 0.002$), CAMCOG memory sub-scale ($r = 0.43$; $p = 0.001$), CAMCOG attention sub-scale ($r = 0.40$; $p = 0.004$), and the right column of the Blessed scale ($r = 0.42$; $p = 0.002$), which basically assesses temporal-spatial orientation, autobiographic memory, short- and long-term recall, and attention skills. Severity of AD as addressed by the CDR score, which was robustly associated with lower MMSE and CAMCOG scores ($p < 0.001$ for both), was further correlated with PLA_2 ($r = -0.36$; $p = 0.03$). Likewise, in the sub-sample of cognitively impaired subjects (i.e., AD and MCI patients only), non-parametric correlation of PLA_2 activity and MMSE scores was significant ($n = 32$, $rho = 0.37$, $p = 0.05$). There were no correlations between PLA_2 activity and the age of onset of dementia or the number of years since the first symptoms of memory impairment.

Discussion

The present study confirms, in an independent sample, our previous findings of reduced platelet PLA_2 activity in AD, and this reduction was correlated with the severity of dementia and with the degree of cognitive impairment. Reduced platelet PLA_2 in AD was not an artefact of treatment with AchE inhibitors or with neuroleptics. Neuroleptic drugs are known to reduce the enzyme activity (Gattaz et al., 1987; Tavares et al., 2003). However, the reduction of PLA_2 activity in AD remained significant after the exclusion of the patients on

neuroleptics from the analysis. Moreover, our finding is not due to a lower number of platelets in AD patients, because our results are corrected and expressed by the amount of protein in the samples.

Numerous PLA_2 subtypes have been so far identified and characterised (Clark et al., 1991; Chen et al., 1994; Hattori et al., 1996; Cupillard et al., 1997; Tang et al., 1997; Underwood et al., 1998). According to biochemical features such as their primary structure, cellular localisation, requirement of calcium (Ca^{2+}), and substrate specificity, these enzymes are classified into several families, including Ca^{2+}-sensitive arachidonoyl-specific 85-kDa citosolic PLA_2 ($cPLA_2$), Ca^{2+}-independent PLA_2 ($iPLA_2$), low molecular weight secretory PLA_2 ($sPLA_2$), and platelet-activating factor-acetylhydrolase (Cupillard et al., 1997).

In the present study, more than 70% of the PLA_2 activity assessed with our assay in platelets refers to the Ca^{2+}-independent form of the enzyme ($iPLA_2$). This is of interest because two isoforms of $iPLA_2$ are also expressed in the brain, preferentially in neurons (Larsson Forsell et al., 1999; Pickard et al., 1999; Diaz-Arrastia and Scott, 1999). Reduced PLA_2 activity has also been reported in different areas of the brain of AD patients, comprising both the Ca^{2+}-dependent and the Ca^{2+}-independent isoforms (Gattaz et al., 1995; Ross et al., 1998; Talbot et al., 2000). Reduced PLA_2 activity in the brain of AD patients is in line with the results of *in vivo* ^{31}P-spectroscopy studies of the parietal cortex (Brown et al., 1989), and recently replicated by our group within the pre-frontal cortex (data in preparation), showing that brain phospholipid turnover is reduced in AD brains. Likewise, a marked reduction in the proportion of docosahexanoate (one of the main free fatty acids released by the deacylation of membrane phospholipids by PLA_2) has been found in the parietal cortex of AD patients (Skinner et al., 1989). Also in the cerebrospinal fluid of AD patients a reduction has been reported of the breakdown of phospatidylcholine into lysophosphatidylcholine, a process in which PLA_2 plays a pivotal role (Mulder et al., 2003).

In face of these data, it is tempting to speculate that reduced PLA_2 activity is a widespread finding in AD, and that the determination of the enzyme activity in platelets might serve as a risk marker for the disease. We are presently testing this hypothesis in individuals with MCI, who represents a high-risk group for AD, as in follow up studies after one year 10% of these individuals will fulfil diagnostic criteria for AD (Petersen et al., 2001). In the present study the mean PLA_2 activity in individuals with MCI was between the values of AD and controls, with a considerable overlap with the AD group. Although the difference between MCI and controls was not statistically significant, we are investigating in a longitudinal study whether lower PLA_2 activity at baseline would predict the switch from MCI to AD during a 4-year follow up.

PLA_2 plays an important role in eicosanoid metabolism and in signal transduction. In tissue culture, it has been shown that arachidonic acid participates in pathways underlying neurite outgrowth and differentiation of neuroblastoma cells (Williams et al., 1994). With respect to AD pathogenesis, arachidonic acid treatment of cultured CHO-m1 cells resulted in stimulation of the secretory pathway of APP metabolism (Emmerling et al., 1996). The stimulation of the secretory pathway of APP reduces the deposition of β-amyloid within the brain.

In fact, cell culture models have demonstrate that the activation of PLA_2 increased the secretion of the amyloid precursor protein (APP), whereas the inhibition of PLA_2 inhibited the carbachol-stimulated secretion of APP from CHO-m1 cells (Emmerling et al., 1993). Thus, we speculate that reduced PLA_2 activity may contribute to brain pathology in AD, affecting membrane phospholipid turnover and arachidonic acid production, which in turn may result in cytoarchitectural changes and contribute to increased β-amyloid deposition within the brain. We are presently investigating the mechanisms by which PLA_2 activity may be reduced in AD, including the determination of circulating inhibitors and the study of PLA_2 genes expression.

Acknowledgements

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