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Increased *in vitro* phosphorylation of a M_r 60,000 protein in brain from patients with Alzheimer disease

(postmortem human brain/protein kinase/tau protein)

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ABSTRACT **We have established *in vitro* conditions under which we can reliably measure kinase activity in normal postmortem human brain. Using these conditions, we detected in the brains of patients with Alzheimer disease a 2-fold increase in the level of M_r 60,000 protein phosphorylation compared to age-matched controls. The M_r 60,000 protein phosphorylation was found exclusively in the cytosol fraction. No differences were detected between phosphoproteins in 100,000 × *g* pellet fractions from brains of Alzheimer disease patients and from age-matched controls. Postmortem time up to 17 hr does not seem to affect the phosphorylation level of the M_r 60,000 protein. Younger Alzheimer disease patients had more prominent changes in the elevation of the M_r 60,000 protein phosphorylation level than older patients, although in the control patient, age did not affect the phosphorylation level of the M_r 60,000 protein. We conclude that in the brain cytosol of Alzheimer disease there may be an abnormality in either the degree of M_r 60,000 protein phosphorylation or in the M_r 60,000 protein concentration.**

Alzheimer disease brings dementia and slow death to >5% of the adult population over the age of 64 yr. There is no clinically reliable diagnostic test for this disease, because clear anatomical and biochemical abnormalities are found only in brain tissue. Despite the accumulating anatomical and biochemical information, the primary abnormality in Alzheimer disease is not known. It is conceivable that the deficit is associated with atrophy of rather specific neurons (1–4). Furthermore, specific neurotransmitter receptors have been reported to be reduced in brains from Alzheimer disease patients (5–9). This loss of receptors is probably associated with degeneration of specific types of neurons. Neurotransmitters for these receptors are associated either with neuritic plaques (10–12) or with neuronal tangles (13). Thus, an obvious and critical question is why cholinergic and other neurons die in the brain of individuals with Alzheimer disease. No biochemical abnormality has been demonstrated to have a causal role in the neuronal degeneration.

The formation of neurofibrillary tangles is one of the major pathologic characteristics of neurons found in the brain of Alzheimer disease patients. It has been shown in tissue sections that perikarya of neurons that bear these tangles contain a phosphorylated form of neurofilament; in contrast, phosphorylated neurofilaments in normal brains are restricted to axons (14). Thus, it is reasonable to suggest that in Alzheimer disease the lesion involves an imbalance of specific kinases responsible for phosphorylation of cytoskeletal elements, including neurofilaments. However, there has been no biochemical study of protein kinases in the brain of Alzheimer disease patients except in a very preliminary form (15, 16). The study of phosphorylation may be all the more important because gene expression in the cell is regulated via regulation of protein phosphorylation. It is reasonable to think that abnormal phosphorylation and eventually abnormal expression of certain proteins may cause degeneration of specific sets of neurons in the brain of individuals with Alzheimer disease.

Here we report the results of an *in vitro* phosphorylation study in which phosphorylation of a cytosolic protein of M_r 60,000 is enhanced in brains of Alzheimer disease patients compared to that in control brains.

MATERIALS AND METHODS

Tissue Preparation. Postmortem human brains were removed and divided sagittally in the midline. The right hemibrain was frozen at −70°C for biochemical analysis, and the left hemibrain was fixed with formalin for neuropathologic diagnosis. All Alzheimer disease samples used in this study had counts of >40 senile plaques per microscopic field (×100) in the midfrontal cortex, which corresponds to the tissue analyzed for phosphorylation from the contralateral hemisphere. All control brains used in this study were confirmed for the absence of senile plaques and neurofibrillary tangles. Preparation of tissue for the biochemical assay was essentially as described previously (17). Midfrontal cortex was dissected from 1-cm-thick sections of frozen tissue on a glass plate cooled from below by a bed of powdered dry ice (18). Dissected cortex was placed in a ×10 volume of homogenization buffer [0.32 M sucrose/5 mM Hepes, pH 8.0/5 mM imidazole/5 mM benzamidine/2 mM 2-mercaptoethanol/3 mM EGTA/0.5 mM $MgSO_4$/5 mM glycerophosphate/5 mM potassium fluoride/0.5 mM $ZnSO_4$/0.1 mM phenylmethylsulfonyl fluoride/leupeptin (0.1 mg/ml)/pepstatin (0.05 mg/ml)/aprotinin (0.1 mg/ml)] and homogenized by two 5-sec strokes of a Polytron homogenizer (Brinkmann). This homogenization buffer was designed to inhibit not only proteases (19) but also phosphoprotein phosphatases (20–24). This homogenate was centrifuged 8 min at 2500 × *g* at 2°C to pellet the nucleus and the majority of cytoskeletal components. The 2500 × *g* supernatant was then centrifuged 1 hr at 100,000 × *g* at 2°C to separate membrane-rich fractions (100,000 × *g* pellet) from cytosol. Pellets were reconstituted to the original volume with the homogenization buffer, aliquoted, frozen in an ethanol/dry ice bath, and stored at −70°C until used for the phosphorylation reaction.

Phosphorylation and $NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Ten microliters of each fraction in the homogenization buffer containing 6.3 μg total protein was mixed with 15 μl of reaction mixture containing 50 mM Tris·HCl, pH 7.6 (all concentrations are for final 25-μl volume), 10 mM $MgSO_4$, 100 μg of protein kinase A inhibitor per ml (Sigma), 5 mM 2-mercaptoethanol, 10 μM ATP [γ-^{32}P]ATP (1 μCi per

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tube; 1 Ci = 37 GBq) (ICN), and 0.2 mM EDTA. The mixture underwent reaction for 12 min at 30°C. The reaction was stopped by addition of 6.3 μl of a buffer containing 40% (vol/vol) glycerol, 25% 2-mercaptoethanol, 12% $NaDodSO_4$, 0.31 M Tris·HCl (pH 6.8), 25 mM EDTA, and 0.1% bromophenol blue. The samples were then subjected to 6.5–12.5% polyacrylamide gel (1-mm thick) electrophoresis with a 2-cm stacking gel (25) at a current of 20 mA per gel. The electrophoresis was stopped when the dye front reached 1 cm from the gel edge. Gels were fixed 1 hr in 10% acetic acid containing 15% isopropyl alcohol, stained 1 hr with 0.2% Coomassie brilliant blue in 10% acetic acid and 40% isopropyl alcohol, and destained in 10% acetic acid and 15% isopropyl alcohol. The destained gel was rinsed 1 hr with water, dried, and exposed ≈10 hr to Kodak X-Omat RP film with a Hi-Plus intensifying screen (DuPont) at −70°C. Protein concentrations were determined by the method of Lowry *et al.* (26) with plasma globulin (Bio-Rad) as the standard. For some experiments, dried gels were dissected and radioactivity was determined by liquid scintillation counting (Tm Analytic, Elk Grove Village, IL).

Staining of Protein Blot with Concanavalin A. For blotting, the separated proteins were electrotransferred from gels to nitrocellulose paper (Millipore) for 2 hr with 8 V/cm in 20% methanol/0.1% $NaDodSO_4$/0.025 M Tris base/0.192 M glycine (pH 8.8) with constant stirring. The nitrocellulose blot was incubated 1 hr in 150 mM NaCl containing 0.1% Tween 20 (Sigma) and 5 mM sodium phosphate (pH 7.4) at room temperature to block nonspecific protein binding site. To detect glycoproteins, blots were exposed to peroxidase-conjugated concanavalin A overnight at 4°C in 2 ml of buffer (0.1% Tween 20/150 mM NaCl/50 mM Tris·HCl, pH 7.4/1 mM $CaCl_2$/0.05% NaN_3), and washed by six changes of the same buffer. The washed blots were placed for 1 min in 50 ml of 20 mM Tris·HCl (pH 7.5) and then in 50 ml of diaminobenzidine solution (25 mg of diaminobenzidine; 50 μl of hydrogen peroxide; 20 mM Tris·HCl, pH 7.5); 500 μl of 1% cobalt chloride was quickly added, and the incubation was continued ≈20 min until staining was visible. The blots were then washed with water and photographed while wet.

RESULTS AND DISCUSSION

$NaDodSO_4$-Soluble Proteins in Brain Tissue as Detected by One-Dimensional $NaDodSO_4$/Polyacrylamide Gel Electrophoresis Are Not Detectably Different in Alzheimer Disease and in Controls. Before studying kinases or kinase substrates by using $NaDodSO_4$/polyacrylamide gels, we must be sure that the $NaDodSO_4$-soluble proteins shown on this one-dimensional gel are not detectably different in Alzheimer disease from those in controls. By studying Coomassie brilliant blue-stained protein patterns in $NaDodSO_4$/polyacrylamide gels of membrane or cytosol fractions, we could not detect any consistent differences between brains from Alzheimer disease and control individuals (Fig. 1). This is consistent with the results reported by other investigators (27, 28). Major glycoproteins in Alzheimer disease brains do not seem to differ from those in control brains either. For comparison of glycoproteins in Alzheimer disease and controls, a protein blot on nitrocellulose paper was stained with peroxidase-conjugated concanavalin A. Again, no consistent difference was observed.

Extent of M_r 60,000 Protein Phosphorylation Is Enhanced in the Brain of Patients with Alzheimer Disease. Comparison of *in vitro* phosphorylation of cytosol showed that the phosphorylation of a protein of M_r 60,000 was more extensive in Alzheimer disease than in control brains (Figs. 1 and 2). The M_r 60,000 protein phosphorylation was detected only in the cytosolic fraction and not in the 100,000 × *g* pellet fraction (Fig. 1). In control brains, phosphorylation of the M_r 60,000

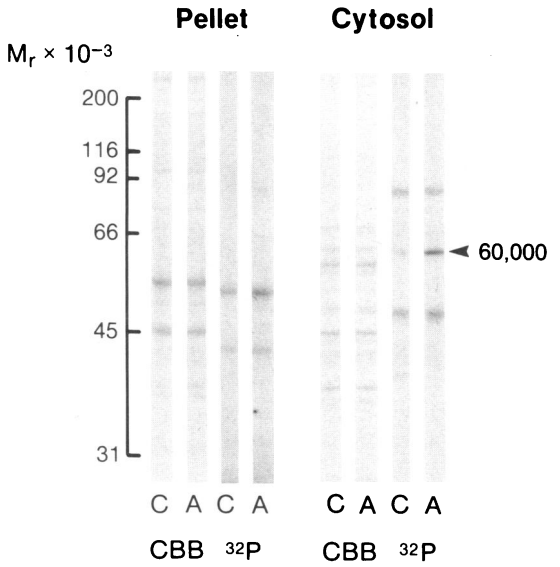

FIG. 1. Enhanced phosphorylation of the cytosolic M_r 60,000 protein in Alzheimer disease brain. Phosphorylation conditions are described in *Materials and Methods*. The Coomassie brilliant blue (CBB) staining of gels with 25 μg of proteins from representative samples as well as the autoradiogram of ^{32}P-labeled proteins (^{32}P) are shown (C, control; A, Alzheimer).

protein comprises 4.1% ± 1.1% (SD) (n = 7) of total cytosolic phosphorylation, whereas in Alzheimer disease it represents 10.3% ± 3.4% (n = 8) (Fig. 2). The difference is significant ($P < 0.002$). Phosphorylation of this protein does not seem to be cAMP, Ca^{2+}/calmodulin, or lipid dependent. In the current study, the phosphorylation reaction mixture routinely included the cAMP-dependent protein kinase inhibitor protein. Elimination of this inhibitor with the concomitant addition of cAMP stimulated the phosphorylation of a number of proteins with molecular weights smaller than 50,000, leaving the M_r 60,000 protein phosphorylation unaffected. Previously, we detected Ca^{2+}/calmodulin-dependent phosphorylation of M_r 65,000 protein and phorbol ester/phosphatidylserine-dependent phosphorylation of M_r 86,000

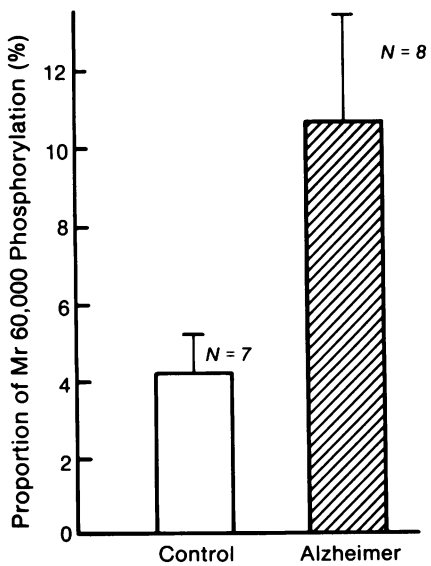

FIG. 2. M_r 60,000 protein phosphorylation in Alzheimer disease brain. Conditions for the phosphorylation reaction as well as the determination of radioactivity are described in *Materials and Methods*. Bar shows the standard deviation. Student's t test shows significant difference ($P < 0.002$).

and M_r 67,000 proteins in postmortem human brains (17). Using the same conditions, we did not see any effect of kinase activators on the M_r 60,000 protein phosphorylation in either control or test samples.

Before concluding altered phosphorylation of a M_r 60,000 protein in brains from Alzheimer disease patients, we need to consider two factors that may confound results obtained with postmortem brains. These two factors are age and postmortem times of brains. Postmortem time for control brains in this report was 7.2 ± 5.5 hr (n = 6; postmortem time for one case could not be obtained) and 7.6 ± 6.6 hr for test brains. Age for control brains was 78 ± 7 yr (n = 7) and 73 ± 6 yr (n = 8) for Alzheimer disease brains. As shown in Fig. 3, the proportion of M_r 60,000 protein phosphorylation was not significantly correlated to the postmortem time. We do not know the postmortem time for one case. This may affect the statistics, because very long postmortem intervals may affect the degree of phosphorylation. Eliminating this case from the statistics, however, does not change our conclusion of increased M_r 60,000 protein phosphorylation in the Alzheimer group (4.3% ± 1.1% in control group vs. 10.2% ± 3.4% in Alzheimer disease patients; $P < 0.002$). Fig. 4 shows that younger patients demonstrate a more marked increase in the M_r 60,000 protein phosphorylation ($r = -0.83$; $P < 0.01$), although no age-dependent change in the M_r 60,000 protein phosphorylation was observed among the control group. It is of interest to note that younger patients have been reported to have more severe Alzheimer disease pathology, such as the loss of nerve cells, a reduction in nucleolar volume, and an increase in the frequency of neuritic plaques and neurofibrillary tangles, than older patients (29, 30). Thus, it is possible that the increased *in vitro* phosphorylation of the M_r 60,000 protein is a biochemical expression of the Alzheimer disease pathology. Taken together, we conclude, although tentatively, that the *in vitro* phosphorylation of a M_r 60,000 protein in the cytosol of patients with Alzheimer disease is enhanced as compared to that in controls.

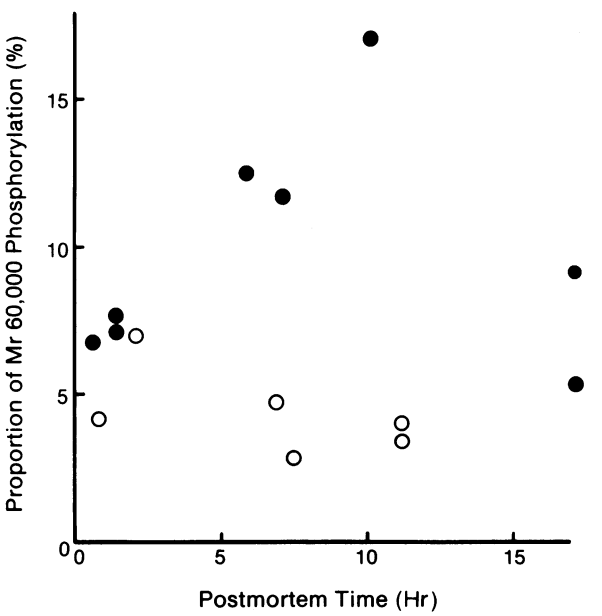

FIG. 3. M_r 60,000 protein phosphorylation as a function of postmortem time. The phosphorylation of the M_r 60,000 protein was determined as described in *Materials and Methods*. The proportion of M_r 60,000 protein phosphorylation as compared to total cytosolic phosphorylation is plotted against postmortem time of individual brain from which specimen was prepared. Points on the figure are from postmortem frontal cortex of different individuals with (●) or without (○) Alzheimer disease. Correlation coefficients for controls and Alzheimer disease groups are $r = -0.65$ and $r = 0.45$, respectively, both of which are not significant.

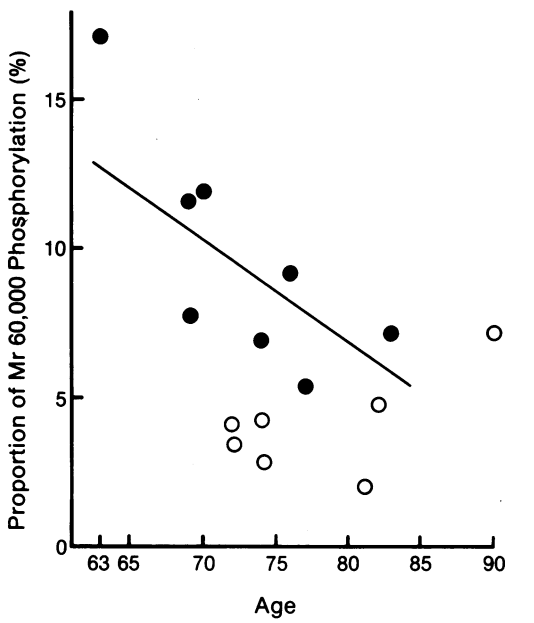

FIG. 4. M_r 60,000 protein phosphorylation as a function of age. Same as Fig. 3, except that the proportion of M_r 60,000 protein phosphorylation is plotted as a function of age of the individual from which specimen was prepared. Correlation coefficients for controls and Alzheimer disease groups are $r = 0.65$ (not significant) and $r = -0.83$ ($P < 0.01$), respectively. A linear regression line is drawn for the Alzheimer disease group.

What could be the molecular explanation for this increased level of M_r 60,000 protein phosphorylation in Alzheimer disease? The simplest explanation would be either increased levels of the M_r 60,000 protein kinase or of the M_r 60,000 protein itself. As is clear from studying Fig. 1, there is no M_r 60,000 protein band adjacent to the M_r 60,000 protein phosphorylated band, indicating that the M_r 60,000 protein is a minor component. Therefore, without having a molecular probe, such as antibody against M_r 60,000 protein, the quantification of this protein is not feasible. As for the M_r 60,000 protein kinase, without having the M_r 60,000 protein substrate there is no method to quantify it. Thus, the purification of the M_r 60,000 protein is a prerequisite for answering these questions.

There are several alternative explanations for our finding of abnormal phosphorylation in Alzheimer disease brains, other than postulating an abnormal kinase or its substrate. Under our conditions of *in vitro* phosphorylation, we detect both the products of the forward reaction (phosphorylation) and the backward reaction (dephosphorylation). It is possible, therefore, that the apparently enhanced phosphorylation of the M_r 60,000 protein *in vitro* is due to increased phosphoprotein phosphatase activity in Alzheimer disease brains and the relative resistance of the M_r 60,000 phosphoprotein to this phosphatase. It may be especially important to test this possibility because it has been reported that Alzheimer disease brain exhibits less kinase activity *in vitro* than control brain (15). However, under our experimental conditions we did not detect reduced levels of *in vitro* phosphorylation in brains of individuals with Alzheimer disease, suggesting that the possibility of increased phosphatase activity is unlikely.

The degree of *in vitro* phosphorylation is proportional to the number of available phosphorylation sites. When endogenous substrates are used, as in this study, it is not the amount of substrate protein that determines the degree of phosphorylation, but the number of unoccupied phosphoryl-

ation sites that were not phosphorylated *in vivo*. Therefore, the apparently enhanced M_r 60,000 protein phosphorylation *in vitro* may reflect less effective phosphorylation of the M_r 60,000 protein *in vivo*, which might be brought about by inhibited kinase activity caused by intoxication of neurons in Alzheimer disease. We postulated that an increased level of phosphorylation of cytoskeletal components causes the dissociation of certain kinases from cytoskeleton to cytosol (31). In Alzheimer disease brains, the phosphorylation state of cytoskeletal components seems to be altered (14, 32). In fact, using histone as a substrate we detected an increased level of kinase activity in cytosol of Alzheimer disease patients with a concomitant decrease in the particulate kinase activity, suggesting the translocation of a kinase (unpublished data). Likewise, it is possible that the compartmentalization of the M_r 60,000 protein kinase is abnormal in Alzheimer disease brains, resulting in altered levels of the M_r 60,000 protein phosphorylation. An alternative possibility may be that in brains of patients with Alzheimer disease there is an altered compartmentalization of the M_r 60,000 protein itself. This protein may be a soluble unassembled form of a molecule normally found predominantly in the insoluble fractions, such as the cytoskeletal fractions that we did not examine in this study.

In this context, it is interesting to note that microtubule-associated protein tau seems to be a major component of paired helical filaments in Alzheimer disease (33–36). The M_r 60,000 phosphoprotein that we detected in this study shares several characteristics with tau protein. (*i*) Their molecular weights are around 60,000, (*ii*) both are phosphoproteins, (*iii*) both are rather basic proteins, and (*iv*) they have a characteristic appearance in Alzheimer disease—increase in the degree of the M_r 60,000 protein phosphorylation and the association of tau protein with paired helical filaments. Isolation and characterization of the M_r 60,000 phosphoprotein and tau protein will hopefully provide an answer to the identity of the M_r 60,000 protein. Whatever the molecular mechanisms of altered *in vitro* phosphorylation of a M_r 60,000 protein, identification of the function of this phosphoprotein, as well as the cellular localization of the protein, will aid in the molecular understanding of cellular abnormalities in Alzheimer disease.

In the current study, we detected an increased degree of M_r 60,000 protein phosphorylation in the frontal cortex of the Alzheimer patient. Neuropathological examination showed that this is the brain region intensely affected by senile plaques, a pathologic marker of Alzheimer disease. One may ask whether the increased level of M_r 60,000 protein phosphorylation is restricted to brain regions affected by Alzheimer disease pathology or if it can be detected in nonaffected organs, such as cerebellum or integument [the systemic nature of Alzheimer disease has been recently demonstrated (37)]. We cannot, so far, detect increased levels of M_r 60,000 protein phosphorylation in fibroblasts from Alzheimer patients. Thus, the increased *in vitro* phosphorylation of the M_r 60,000 protein seems to be closely related to Alzheimer disease pathology, such as the formation of neurofibrillary tangles, and not the biochemical expression of the systemic changes in Alzheimer disease.

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