Communication

Nontoxic Amyloid β Peptide1–42 Suppresses Acetylcholine Synthesis

POSSIBLE ROLE IN CHOLINERGIC DYSFUNCTION IN ALZHEIMER’S DISEASE*

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We show here that amyloid β peptide1–42 (Aβ1–42) may play a key role in the pathogenesis of the cholinergic dysfunction seen in Alzheimer’s disease (AD), in addition to its putative role in amyloid plaque formation. Aβ1–42 freshly solubilized in water (non-aged Aβ1–42), which was not neurotoxic without preaggregation, suppressed acetylcholine (ACh) synthesis in cholinergic neurons at very low concentrations (10–100 nM), although non-aged Aβ1–40 was ineffective. Non-aged Aβ1–42 impaired pyruvate dehydrogenase (PDH) activity by activating mitochondrial protein kinase Iglycogen synthase kinase-3β, as we have already shown in hippocampal neurons (Hoshi, M., Takashima, A., Naguchi, K., Murayama, M., Sato, M., Kondo, S., Saitoh, Y., Ishiguro, K., Hoshino, T., and Imahori, K. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2719–2723). Neither choline acetyltransferase activity nor choline metabolism was affected. Therefore, the major cause of reduced ACh synthesis was considered to be an inadequate supply of acetyl-CoA owing to PDH impairment. Soluble Aβ1–42 increases specifically in AD brain (Kuo, Y.-M., Emmerling, M. R., Vigo-Pelfrey, C., Kasunic, T. C., Kirkpatrick, J. B., Murdoch, G. H., Ball, M. J., and Roher, A. E. (1996) J. Biol. Chem. 271, 4077–4081). This increase in soluble Aβ1–42 may disturb cholinergic function, leading to the deterioration of memory and cognitive function that is characteristic of AD.

Alzheimer’s disease (AD) is a progressive dementia. One of the most consistent abnormalities in AD brain is a severe loss of basal forebrain cholinergic neurons and cortical cholinergic innervations (3–11), together with other pathological features (amyloid plaques and neurofibrillary tangles) (12). The degree of cognitive dysfunction in AD patients is significantly correlated with decline in choline acetyltransferase (ChAT) activity, a cholinergic marker, and loss of cholinergic neurons (7, 13–15). Evidence from behavioral, pharmacological, neurochemical, and lesion studies further supports a role of these cholinergic deficits in the memory disturbances in normal aging and AD (16, 17). Cholinergic therapies based on acetylcholine esterase (AChE) inhibition have produced small, but well-attested improvements in AD patients (16). These results suggest a critical involvement of cholinergic deficiency in the impaired learning and memory in AD. However, the basis for this cholinergic deficiency is unclear, and whether amyloid plaques or neurofibrillary tangles are related to the cholinergic deficits is so far unknown.

Amyloid plaques are mainly composed of insoluble aggregates of amyloid β peptides (Aβs). In our previous work, aggregated Aβ25–35, an active portion of Aβ, was shown to suppress pyruvate dehydrogenase (PDH) activity in hippocampal neuronal cultures and to result in energy failure, which probably contributes to neuronal death, together with abnormal phosphorylation of τ (1). Since PDH provides acetyl-CoA for acetylcholine (ACh) synthesis in cholinergic neurons, the above finding suggests the direct involvement of Aβ in the cholinergic abnormality in AD by inhibiting PDH activity. Among Aβ species generated in vivo from amyloid precursor protein (APP), Aβ1–40 is a major product of soluble Aβs and is constitutively secreted in culture medium and cerebrospinal fluid, while Aβ1–42 is produced in a lesser amount (18, 19). Accumulating evidence indicates that increased production of Aβ1–42 is the primary event that leads to the formation of amyloid plaques in AD. Therefore, we focused on the effect of Aβ1–42 on cholinergic function using a primary neuronal culture as an in vitro model system. We report here that Aβ1–42 freshly solubilized in water impairs ACh synthesis in cholinergic neurons without affecting neuronal survival. Recently, the pool of oligomeric water-soluble Aβ1–42 was found to be uniquely elevated in AD compared with normal brain (2). Taken together, the findings suggest a possible role of soluble Aβ1–42 in AD pathogenesis in addition to its contribution to amyloid plaque formation.

EXPERIMENTAL PROCEDURES

Peptides—Synthetic Aβ1–40 peptide was synthesized and purified as described previously (20). Synthetic Aβ1–42 peptide was obtained from Bachem (Torrance, CA). Lyophilized peptides were stored at −20 °C. Non-aged Aβs were prepared by freshly dissolving the lyophilized peptides in autoclaved MilliQ water to 200 μM just before use. Aged Aβ1–42 was produced by dissolving the peptide in autoclaved MilliQ water to 350 μM and incubating at 37 °C for 7 days as previously reported (21).

Cell Culture—Primary cultures from rat septum regions, which include septal and basal forebrain cholinergic neurons, were prepared and plated (1). After 3 days, the medium was changed to serum-free neurobasal medium with B27 and l-glutamate supplements (Life Technologies, Inc.). Two days later, non-aged Aβ was added to the medium.

Biochemical Assays—Intracellular ACh was determined using an HPLC-electrochemical detector system (1, 22). Rate of ACh synthesis from [2-14C]pyruvate was measured by the modified method of Gibson et al. (23). Cultures were incubated with various concentrations of non-aged Aβ1–42 for 12 h, and [2-14C]pyruvate (1 μCi/ml) was added to the medium. After a 40-min incubation, cultures were washed with PBS and extracted with 5% TCA/Trit-CI (pH 7.5), 0.5% Triton X-100, and scraped off. Supernatants were recovered by centrifugation, and ACh was extracted by adding 14 μl sodium phosphate buffer (pH 7.4) containing sodium tetrathylborate and acetonitrile, followed by tolune-based scintillant. Incorporation of radioactivity from [2-14C]pyruvate into cognitive dysfunction in AD patients is significantly correlated with decline in choline acetyltransferase (ChAT) activity, a cholinergic marker, and loss of cholinergic neurons (7, 13–15). Evidence from behavioral, pharmacological, neurochemical, and lesion studies further supports a role of these cholinergic deficits in the memory disturbances in normal aging and AD (16, 17). Cholinergic therapies based on acetylcholine esterase (AChE) inhibition have produced small, but well-attested improvements in AD patients (16). These results suggest a critical involvement of cholinergic deficiency in the impaired learning and memory in AD. However, the basis for this cholinergic deficiency is unclear, and whether amyloid plaques or neurofibrillary tangles are related to the cholinergic deficits is so far unknown.

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vate into ACh was proportional to time, and the rate of ACh synthesis was calculated as cpm/min. ChAT activity was assayed as described (1). ATP content in the culture was measured by luciferase reaction using a Promega kit (FF2000 and FF2040) with a luminometer, Lumat LB9501/16 (Berthold Japan, Tokyo). AChE activity was determined in terms of production of [14C]acetyl-CoA from [2-14C]pyruvate with an excess amount of ChAT, which converted the produced [14C]acetyl-CoA into [14C]ACh. After non-aged Aβ1–42 treatment, cultures were washed with PBS, scraped off in 10 mM potassium phosphate buffer (pH 7.4), 1 mM EGTA, 1 mM EDTA, and 0.1% Triton X-100, frozen, and thawed. This homogenate was incubated at 37 °C for 40 min in the standard reaction buffer for PDH activity (1) (50 mM potassium phosphate buffer (pH 8.0), 2.5 mM NAD, 0.2 mM thiamin pyrophosphate, 0.13 mM CoA, and 2.6 mM L-cysteine) containing 1 mM choline chloride, ChAT (Sigma, C-3388, 0.05 unit/assay), and 1 mM [2-13C]pyruvate (0.5 μCi/assay). The reaction was terminated by adding 14 mM sodium phosphate buffer (pH 7.4) containing sodium tetrathionate and ascorbate, followed by toluene-based scintillant. PDH activity was measured as production of [14C]acetyl-CoA from [2-14C]pyruvate with an excess amount of ChAT, which converted the produced [14C]acetyl-CoA into [14C]ACh. Non-aged Aβ1–42 treatment, cultures were washed with PBS, scraped off in 10 mM potassium phosphate buffer (pH 7.4), 1 mM EGTA, 1 mM EDTA, and 0.1% Triton X-100, frozen, and thawed. This homogenate was incubated at 37 °C for 40 min in the standard reaction buffer for PDH activity (1) (50 mM potassium phosphate buffer (pH 8.0), 2.5 mM NAD, 0.2 mM thiamin pyrophosphate, 0.13 mM CoA, and 2.6 mM L-cysteine) containing 1 mM choline chloride, ChAT (Sigma, C-3388, 0.05 unit/assay), and 1 mM [2-13C]pyruvate (0.5 μCi/assay). The reaction was terminated by adding 14 mM sodium phosphate buffer (pH 7.4) containing sodium tetrathionate and ascorbate, followed by toluene-based scintillant.

**AChE Cytochemistry—** AChE staining was performed as described (26). Briefly, after aged Aβ1–42 or non-aged Aβ1–42 treatments at 10 μM for 24 h, cultures were washed with PBS, fixed for 30 min with 4% paraformaldehyde at room temperature, washed with PBS, and then incubated for 3 days at 4 °C in 50 mM acetate buffer (pH 5.0) containing 4 mM acetyltiothiocholine iodide, 2 mM copper sulfate, 10 mM glycine, and 10 mg/ml gelatin. Non-specific cholinesterases were inhibited by addition of 0.2 mM ethopropagine in the above medium. Then, the gelatin was dissolved by incubation at 37 °C. Cultures were washed with water, exposed for 1 min to 1.25% NaNO3, washed with water, and exposed for 1 min to 1% AgNO3.

**Assay for τ Protein Kinase 1/Glycogen Synthase Kinase-3β (TPKI/GSK-3β) Activity—** After non-aged Aβ1–42 treatment, mitochondrial pellets were prepared from the culture (1) and disrupted in a buffer (10 mM Tris-Cl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 50 mM NaCl, 50 mM NaF, 50 mM β-glycerophosphate, 0.5 mg/ml benzamidine, 1 μg/ml an-tipain and leupeptin, 30 μM okadaic acid, 1 μM phenylmethylsulfonyl fluoride, 1 μM vanadate, and 0.5% Tween 20). TPKI/GSK-3β activity was determined by an immunoprecipitation assay using modified phosphoglycogen synthase peptide as a substrate (1).

**RESULTS**

**Freshly Dissolved Aβ1–40 and Aβ1–42 (Non-aged Aβ1–40 and Non-aged Aβ1–42) Are Not Neurotoxic—** Primary septal cultures have been established from rat septum regions that include septal and basal forebrain cholinergic neurons (1). Aβ is normally secreted in vivo as a soluble form, which aggregates into insoluble amyloid fibrils to form amyloid plaques (21, 27). Synthetic Aβ required preincubation for 2–7 days at 37 °C to produce insoluble amyloid fibrils, which were cytotoxic to septal cultures (Fig. 1A, B, and F), as is consistent with the previous report (21). Here, we use synthetic Aβ1–40 and Aβ1–42 peptides solubilized freshly in distilled water, which we designate as non-aged Aβ1–40 and non-aged Aβ1–42, respectively. Non-aged Aβ1–40 did not present any toxicity to septal cultures. Since soluble Aβ1–42 is known to aggregate into insoluble, toxic amyloid fibrils more rapidly than Aβ1–40 (28), we have examined the cytotoxicity of non-aged Aβ1–42 at concentrations up to 10 μM. Primary cultures treated with non-aged Aβ1–42 at 10 μM for 24 h (Fig. 1C) were morphologically indistinguishable from the control (Fig. 1A). No difference was observed between the control culture and the culture treated with non-aged Aβ1–40 for 48 h (data not shown). The cellular ATP level was stable after non-aged Aβ1–42 treatment for 12 h (Fig. 1D), indicating that non-aged Aβ1–42 at concentrations up to 10 μM did not disturb the energy metabolism in the culture. Histochemical staining for AChE, a marker enzyme of cholinergic neurons, revealed that cholinergic neurons were not affected by non-aged Aβ1–42 treatment at 10 μM for 24 h (Fig. 1, E and G). ChAT activity, another marker of cholinergic neurons, was not decreased by exposure of the neurons to non-aged Aβ1–42 for 12 h (Fig. 1H). Thus, non-aged Aβ1–42 was not cytotoxic to cholinergic neurons in culture. These results suggest that neither non-aged Aβ1–42 nor non-aged Aβ1–40 used in our experiments formed toxic amyloid fibrils.

**Non-aged Aβ1–42 Suppressed ACh Synthesis in the Culture, Non-aged Aβ1–40 Did Not—** Primary septal cultures were treated with either non-aged Aβ1–40 or non-aged Aβ1–42 for 12 h, and the intracellular ACh level was determined by means of HPLC with an electrochemical detector system (1). Non-aged Aβ1–42 at 10 nm reduced the intracellular ACh level to ~40% of the control after a 12-h treatment, whereas non-aged Aβ1–40 was ineffective (Fig. 2A). Since neither non-aged Aβ1–40 nor non-aged Aβ1–42 was neurotoxic, cholinergic neuronal loss cannot be the cause of the reduced ACh level. Therefore, the decline in the intracellular ACh level results either from reduced ACh synthesis or from accelerated ACh release. Non-aged Aβ1–42 at 10 nm caused a
Effect of Nontoxic Aβ<sub>1–42</sub> on Cholinergic Neurons

**Fig. 2. Suppression of ACh synthesis in cholinergic neurons by physiological concentrations of non-aged Aβ<sub>1–42</sub>**. A, primary septal cultures were treated with various concentrations of either non-aged Aβ<sub>1–42</sub> or non-aged Aβ<sub>1–42</sub> for 12 h, and intracellular ACh was determined by using an HPLC-electrochemical detector system. B, after a 12-h incubation of cultures with non-aged Aβ<sub>1–42</sub>, the ACh synthesis rate was determined by measuring transfer of radioactivity from [2-<sup>14</sup>C]pyruvate per min. Values are means of three determinations ± S.D.

Significant reduction in the intracellular ACh level (Fig. 2A). A corresponding reduction was induced in the synthesis rate of ACh from pyruvate by treatment with non-aged Aβ<sub>1–42</sub> at 10 nM for 12 h (Fig. 2B). Thus, reduced ACh synthesis is primarily responsible for the decline in the intracellular ACh level after non-aged Aβ<sub>1–42</sub> treatment. Treatment of non-aged Aβ<sub>1–42</sub> at zero time affected neither the intracellular ACh level nor the ACh synthesis rate. The results suggest Aβ<sub>1–42</sub> remaining in the cell lysate has no direct effect on the assay procedures, but non-aged Aβ<sub>1–42</sub> works on the living neurons to disturb the ACh synthesis.

**Suppression of ACh Synthesis Can Be Attributed to Reduced Mitochondrial PDH Activity, Probably via TPKI/GSK-3β Activation by Non-aged Aβ<sub>1–42</sub>**—ACh is synthesized from choline and acetyl-CoA by ChAT, which is not rate-limiting under normal conditions (29). Treatment with non-aged Aβ<sub>1–42</sub> for 12 h did not change ChAT activity (Figs. 1H and 3A). Therefore, a reduced supply of either choline or acetyl-CoA accounts for the decline in ACh synthesis.

Neurons obtain choline mainly from the diet using a specialized transporter system that also salvages free choline generated from ACh released intrasynaptically by AChE (30). Treatment with non-aged Aβ<sub>1–42</sub> did not interfere with this choline uptake system, since we found no statistically significant change in the choline uptake in the case of non-aged Aβ<sub>1–42</sub> treatment (Fig. 3A). In accordance with AChE cytochemistry (Fig. 1, E and G), ChAT activity was stable after non-aged Aβ<sub>1–42</sub> treatment for 12 h (Fig. 3A). These results suggest that non-aged Aβ<sub>1–42</sub> did not influence choline turnover in cholinergic neurons. This implies that a reduced supply of acetyl-CoA from pyruvate must be critical (Fig. 2B).

In adult mammalian brain, acetyl-CoA is mainly produced by oxidative decarboxylation of pyruvate by mitochondrial PDH complex. Non-aged Aβ<sub>1–42</sub> at a saturating concentration of 100 nM reduced PDH activity to ~30% of the control (Fig. 3B), which corresponds well with the dose-dependent reductions of the intracellular ACh level (Fig. 2A) and ACh synthesis rate (Fig. 2B). Non-aged Aβ<sub>1–42</sub> treatment for 3 h was enough to cause maximal inhibition of PDH activity in the culture. Correspondingly, the intracellular ACh reached the lowest level after non-aged Aβ<sub>1–42</sub> exposure for 3 h. Thus, it appears that non-aged Aβ<sub>1–42</sub> suppressed ACh synthesis mainly by inhibiting PDH activity and so reducing the availability of acetyl-CoA.

PDH activity is regulated by phosphorylation (31, 32). We have previously found that PDH is phosphorylated and inactivated by TPKI/GSK-3β in vitro as well as in Aβ<sub>25–35</sub>-treated hippocampal neurons (1). In septal cultures, 10 nM Aβ<sub>1–42</sub> was enough to activate mitochondrial TPKI/GSK-3β by 2-fold (Fig. 3B). These results suggest that TPKI/GSK-3β is the main mediator of the inactivation of PDH by non-aged Aβ<sub>1–42</sub> in cholinergic neurons, as previously found in hippocampal neurons.

**DISCUSSION**

Aβ<sub>1–42</sub>, a minor species in normal APP metabolism, is highly aggregable (28), acts as a seed for amyloid fibrils (33), and seems to deposit initially in amyloid plaques in AD (34) and Down’s syndrome (DS) (35). APP mutation linked to familial AD (FAD) or an increased APP gene dosage in DS brain leads to a specific increase in Aβ<sub>1–42</sub> production (19, 36). Recently, Younkin and colleagues reported that mutations in FAD genes, including presenilin 1 (37) and presenilin 2 (38), result in increased levels of Aβ<sub>1–42</sub> in fibroblasts and plasma in some FAD kindreds (39). These studies indicate a critical and central role for Aβ<sub>1–42</sub> in amyloid plaque formation in AD. The findings...
presented here reveal a new aspect of \( \mathrm{A\beta}_{1-42} \) function, implying a role in inducing cholinergic dysfunction, besides amyloid plaque formation.

In the present work, we used \( \mathrm{A\beta}_{1-42} \) freshly dissolved in water (non-aged \( \mathrm{A\beta}_{1-42} \)), which exhibited no neurotoxicity and was confirmed to be in an oligomeric state by laser light-scattering analysis (data not shown). Therefore, the non-aged \( \mathrm{A\beta}_{1-42} \) used in our experiments probably does not form toxic amyloid fibrils, but does impair ACh synthesis in cholinergic neurons at very low concentrations (10–100 nm). Soluble \( \mathrm{A\beta}_{1-42} \) production is uniquely elevated in brains of AD (2) and DS patients before amyloid plaque formation (36), suggesting a role of soluble \( \mathrm{A\beta}_{1-42} \) in the pathogenesis. We hypothesize that soluble \( \mathrm{A\beta}_{1-42} \) produced at an early stage of AD starts affecting cholinergic neurons by suppressing ACh synthesis, causes a reduction in ACh release, modulates synaptic connections, and finally results in cholinergic deficits, which may induce progressive loss of memory and cognitive function in AD patients. Thus, nontoxic, soluble \( \mathrm{A\beta}_{1-42} \) may play a primary role in the cholinergic dysfunction of AD patients by suppressing ACh synthesis, besides contributing to amyloid plaque formation.

TPK/GSK-3\( \beta \) is present both in cytoplasm and in mitochondria in neurons (40). We found that in cholinergic neurons non-aged \( \mathrm{A\beta}_{1-42} \) at 10 nm activates mitochondrial TPK/GSK-3\( \beta \), which mediates the reduction in ACh synthesis via PDH inactivation, as we had previously shown in hippocampal neurons (1). One critical difference between the cholinergic and hippocampal systems lies in the energy metabolism. In the case of hippocampal neurons, activation of mitochondrial TPK/GSK-3\( \beta \) by aggregated \( \mathrm{A\beta}_{25-35} \) inactivates PDH, which causes the energy depletion (1). Hippocampal neurons may die as a result of insufficient energy supply, as well as abnormally phosphorylated \( \tau \) that impairs axonal transport (41). However, in the case of cholinergic neurons, inactivation of PDH activity by non-aged \( \mathrm{A\beta}_{1-42} \) does not interfere with the energy metabolism and the neurons survive. We speculate that the anaplerotic pathway between glycolytic breakdown and the tricarboxylic acid cycle functions in cholinergic neurons, but aggregated \( \mathrm{A}\beta \) inhibits this pathway in hippocampal neurons. Whether the difference in the energy metabolism depends on the \( \mathrm{A}\beta \) species or on the neuronal species needs to be clarified.

Based on the present study, we suggest that \( \mathrm{A\beta}_{1-42} \) participates directly in the induction of cholinergic deficiency, as well as amyloid plaque formation in AD. Application of the findings presented here as a hypothetical in vitro model for AD pathogenesis should open up many possibilities for further research.

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