Regulation of mitochondrial pyruvate dehydrogenase activity by tau protein kinase I/glycogen synthase kinase 3β in brain

(Alzheimer disease/β-amyloid peptide/neuronal death/acytcholine)

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ABSTRACT According to the amyloid hypothesis for the pathogenesis of Alzheimer disease, β-amyloid peptide (βA) directly affects neurons, leading to neurodegeneration and tau phosphorylation. In rat hippocampal culture, βA exposure activates tau protein kinase I/glycogen synthase kinase 3β (TPKI/GSK-3β), which phosphorylates tau protein into Alzheimer disease-like forms, resulting in neuronal death. To elucidate the mechanism of βA-induced neuronal death, we searched for substrates of TPKI/GSK-3β in a two-hybrid system and identified pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl-CoA in mitochondria. PDH was phosphorylated and inactivated by TPKI/GSK-3β in vitro and also in βA-treated hippocampal cultures, resulting in mitochondrial dysfunction, which would contribute to neuronal death. In cholinergic neurons, βA impaired acetylcholine synthesis without affecting choline acetyltransferase activity, which suggests that PDH is inactivated by βA-induced TPKI/GSK-3β. Thus, TPKI/GSK-3β regulates PDH and participates in energy metabolism and acetylcholine synthesis. These results suggest that TPKI/GSK-3β plays a key role in the pathogenesis of Alzheimer disease.

Histopathological features of Alzheimer disease (AD) include extracellular deposits of amyloid fibrils in the cores of senile plaques (1), intracellular neurofibrillary tangles that are composed of paired helical filaments (2–5), and extensive neuronal loss. The mechanisms that lead to AD pathology are not clear. However, the discovery that mutations in the gene for amyloid precursor protein linked to familial AD (6, 7) supports the amyloid hypothesis (8), which claims that β-amyloid peptide (βA) deposition directly contributes to neurodegeneration and the formation of neurofibrillary tangles in AD brain. In primary rat hippocampal cultures, βA exposure induces neuronal degeneration as well as aberrant tau protein phosphorylation, affording a product similar in character to paired helical filament-tau (9, 10). We first purified protein kinases from the microtubule fraction in bovine brain (11). One of these enzymes was named tau protein kinase I (TPKI) and was identical to the β isoform of glycogen synthase kinase 3 (GSK-3β) (12–14). This enzyme modified normal tau protein to a highly phosphorylated form bearing an epitope of paired helical filament and exhibiting a large shift in electrophoretic mobility, both in vitro (11) and in cells in which tau protein and TPKI/GSK-3β were overexpressed (15). By using an antisense oligonucleotide, we recently obtained evidence that TPKI/GSK-3β is involved in tau protein phosphorylation and neuronal death (9). On the basis of these results, our hypothesis to explain βA-induced neuronal death is that βA interacts with neurons and increases the TPKI/GSK-3β level. The increase in the kinase activity leads to extensive phosphorylation of tau protein and destabilization of microtubules, resulting in neuritic dystrophy, impaired axonal transport, and possibly neuronal death. Indeed, phosphorylation of tau protein by βA-activated TPKI/GSK-3β precedes the accumulation of amyloid precursor protein through disruption of axonal transport, and the amyloid precursor protein accumulation contributes to βA-induced neuronal death (16). Nevertheless, the possibility is not yet excluded that another substrate for TPKI/GSK-3β may be partly or wholly responsible for the neuronal death in hippocampal cultures because, for example, antisense oligonucleotides for amyloid precursor protein were less potent protectors of neurons than those for TPKI/GSK-3β in cultures exposed to βA (16). We supposed that some substrate of TPKI/GSK-3β other than tau is also implicated in neuronal death.

In this study, we surveyed TPKI/GSK-3β-interacting proteins from a human brain cDNA library using a yeast two-hybrid system (17). One of the candidate substrates for TPKI/ GSK-3β was pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl CoA in mitochondria. TPKI/GSK-3β phosphorylated this enzyme and inhibited its activity in vitro and also in βA-treated hippocampal culture. Inactivation of PDH by βA exposure resulted in dysfunction of mitochondria, contributing to neuronal death through failure of energy metabolism and leading to a reduced level of acetylcholine (ACh) in cholinergic neurons owing to decreased acetyl-CoA production. These results provide evidence that TPKI/GSK-3β acts not only as tau kinase, but also as PDH kinase, and may offer additional insight into the pathogenesis of AD.

MATERIALS AND METHODS

Plasmid Construction. The expression plasmid pGBT9-TPKI/GSK-3β was constructed by inserting a DNA fragment harboring the TPKI/GSK-3β coding region into the SalI site of pGBT9 (Clontech), resulting in in-frame fusion of TPKI/GSK-3β protein to the C terminus of the GAL4 DNA-binding domain.

Yeast Two-Hybrid Screening. Two-hybrid screening for TPKI/GSK-3β interacting proteins was done as described (17) using a Matchmaker two-hybrid system (Clontech). The yeast strain used was HF7c (MATa, ura3-52, his3-200, lys2-801, ade2-101, trpl-901, leu2-3, 112, gal4-542, gal80-538, LYS::GAL1-HIS3, URA3::(GAL4 17-mers)-3CYC1-lacZ). Screening was done with a 37-year-old human whole-brain cDNA library tagged with GAL4 transcriptional activation domain (Clontech; HL4004AB). Transformants were plated on Leu-, Trp−, His− medium and further screened by the filter

Abbreviations: AD, Alzheimer disease; βA, β-amyloid peptide; TPK, tau protein kinase; GSK-3, glycogen synthase kinase 3; PDH, pyruvate dehydrogenase; ACh, acetylcholine; ChAT, choline acetyltransferase; LDH, lactate dehydrogenase; X-Gal, 5-bromo-4-chloro-3-indolyl β-d-galactoside.

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5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) assay. The rescued cDNA plasmids were recovered from His+ β-galactosidase-positive transformants and cotransformed back into HF7c with pGBT9, so as to identify those cDNA isolates specifically positive with TPKI/GSK-3β. Then the sequences of these cDNA plasmids were analyzed by using the GAL4 activation domain junction primer (Clontech).

**Cell Culture.** Primary rat hippocampal and septum cultures from embryonic day-18 fetuses (9) were plated at a density of 2 × 10^5 cells per cm² on polylysine-coated plates in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (HyClone). After 3 days, the medium was changed to serum-free neurobasal medium (GIBCO) with B-27 (GIBCO) and L-glutamate (GIBCO) supplements. Experiments were done by using the culture at 6 to 7 days. βAs (25–35) were synthesized and purified as described (9). βA stock solution (2 mM in water) was frozen at −20°C for >24 hr and thawed at 37°C. Turbid freeze-thawed βA solution was used for each experiment.

**In Vitro Phosphorylation of PDH by TPKI/GSK-3β.** The α subunit of PDH was purified from porcine heart PDH complex (Sigma) according to the method described by Matuda et al. (1). The kinase-inactivated PDH complex (pellet) was prepared by pretreatment of PDH complex purified from porcine heart (Sigma) with 5 mM dichloroacetate. A specific PDH kinase inhibitor (19), for 10 min at 37°C in kinase buffer containing 100 mM MES (pH 6.5), 0.9 mM magnesium acetate, 1 mM EGTA, 0.02% Tween 20, 10% (vol/vol) glycerol, and 5 mM 2-mercaptoethanol. TPKI/GSK-3β and TPKII were purified from bovine brain (11). The purified α subunit of PDH (0.1 µg) was incubated at 30°C with or without TPKI/GSK-3β for 30 min in kinase buffer containing 0.5 mM ATP. The kinase-inactivated PDH complex (4.7 milliunits) was incubated with or without TPKI/GSK-3β or TPKII at 30°C for the indicated periods under the above condition. PDH activity was assayed by monitoring NADH production at 340 nm (20) in the reaction buffer (50 mM potassium phosphate buffer, pH 8.0/2.5 mM NAD/0.2 mM thiamine pyrophosphate/0.13 mM CoA/2.6 mM L-cysteine/5 mM pyruvate/enzyme source) using an EL340 automated microplate reader (Bio-Tek, Burlington, VT).

**Immunoblotting.** Subcellular fractionation of hippocampal neuronal cultures was performed by differential centrifugation according to the method described by McKeel and Jarett (21) as described (22). Briefly, cell pellets were homogenized in a buffer (10 mM potassium phosphate, pH 7.4/1 mM EGTA/1 mM EDTA/5 mM 2-mercaptoethanol/1 mM phenylmethyl-sulfonil fluoride/0.25 M sucrose). This crude homogenate (Homogenate) was centrifuged (10,000 × g, 10 min) to remove undisrupted cells and nuclear fraction; about 30% of the TPKI/GSK-3β immunoreactivity included in the crude homogenate was removed by the centrifugation; however, the remaining 70% was exclusively recovered in fractions presented in Fig. 2A. After centrifugation (13,000 × g, 5 min) to remove particulate fraction, the supernatant was recovered as Cytoplasm, and the pellet (particulate fraction) was washed twice in the same buffer solution by centrifugation (13,000 × g, 5 min), resuspended, and centrifuged (600 × g, 5 min). The resulting supernatant was centrifuged (13,000 × g, 5 min). The supernatant was called as Supernatant, and the pellet was resuspended in the extraction buffer (50 mM potassium phosphate, pH 7.0/1 mM EGTA/1 mM EDTA/5 mM 2-mercaptoethanol/1 mM phenylmethylsulfonil fluoride/0.1% Tween 20) to comprise mitochondria (Mitochondria). Samples (10 µg per lane) taken at each step were separated on SDS/10% PAGE and electropho-assayed onto nitrocellulose membranes (Schleicher & Schuell). Each membrane was incubated with polyclonal antibody against the C-terminal peptide of TPKI/GSK-3β (C-1) (23) (1:800) followed by 125I-labeled protein A (0.1 µCi/ml; 1 Ci = 37 GBq). Immunoreactive bands were visualized and quantitated by laser densitometry (Fuji BAS 2000).

**Immunoelectron Microscopic Analyses.** Ultrathin sections of hippocampus were prepared from a freshly isolated 27-day-old rat brain as described (24). Immunogold detection of the antigen was done as described (22). Briefly, incubation with the first antibody C-1 was performed for 1 hr at room temperature. After extensive washing with phosphate-buffered saline/1% bovine serum albumin, the second antibody reaction (10 nm gold-labeled goat-anti-rabbit IgG, Amersham; 1:20 dilution) was performed for 1 hr at room temperature. Tissues were washed, contrasted with 2% uranyl acetate for 8 min, rinsed with distilled water, dried, and studied in a JEM-1200EX electron microscope (Joel).

**In Vivo PDH Inactivation After βA Treatment.** Homogenates from hippocampal neuronal cultures for PDH assay were prepared by two methods. (i) Cell pellets were incubated in the extraction buffer for 5 min and centrifuged. Pellets were homogenized, and supernatants were recovered after centrifugation (18,000 × g, 60 min; Beckman model TLA 100.3). (ii) Mitochondrial pellets were prepared from cells as described (21, 22) and disrupted in extraction buffer for 20 sec using a sonicator. PDH activities in these homogenates were assayed by monitoring NADH production at 340 nm, except that pyruvate-dependent NADH production was obtained by subtracting the value of each sample in the same reaction buffer without pyruvate. Lactate dehydrogenase (LDH) was almost completely removed from the above homogenates. Perturbation of PDH activity by residual LDH was similar in each sample, though βA slightly reduced total LDH.

**Assay for TPKI/GSK-3β and TPKII Kinase Activities.** TPKI/GSK-3β and TPKII kinase activities were measured by means of an immunoprecipitation assay (25). We used the modified phosphoglyceren synthase phosphate (phospho-Ala3β-GS-1) in which serine at site 3b is changed to alanine as a TPKI/GSK-3β substrate, and pro-Src peptide (26) as a TPKII substrate.

**Biochemical Assays.** To assay intracellular ACh, cell pellets from septum neuronal cultures were sonicated in ice-cold 0.1 M perchloric acid/0.1% EDTA solution for 1 min. Proteins were removed by centrifugation. Pellets were dissolved in NaOH solution and then neutralized with HCl; protein concentrations were determined by using a Bio-Rad protein assay kit (27). After addition of saturated K₂CO₃ (final 10%), the supernatants were chilled on ice for 30 min, and centrifuged briefly. The supernatants were recovered, neutralized with HCl, filtered through a 0.22-µm membrane filter, and assayed for ACh with an HPLC-electrochemical detector system (28) using an immobilized-enzyme packed column (BAS-Tokyo, Japan). The determination of choline acetyltransferase (ChAT) activity was performed by Fonnun’s method (29).

**RESULTS**

**Screening of TPKI/GSK-3β-Interacting Proteins.** To identify TPKI/GSK-3β-interacting proteins in a yeast two-hybrid system (17), we constructed a plasmid coding the fusion protein between TPKI/GSK-3β and GAL4 DNA-binding domain. The plasmid was transformed into yeast, and the expressed GAL4-TPKI/GSK-3β hybrid protein was shown to retain the kinase activity (specific activity; 2–10 pmol/min per mg of yeast protein) in an immunoprecipitation assay (25) using phospho-Ala3b-GS-1 as a substrate. This hybrid was used to screen a 37-yr-old-human whole-brain cDNA library tagged with GAL4 transcriptional activation domain (Clontech; HL4004AB). Interacting proteins were detected as TPKI/GSK-3β-dependent expression of two reporter genes (His₃ and LacZ). By screening >4 × 10⁶ transformants, we obtained three independent clones encoding PDH, a component of the mitochondrial multienzyme PDH complex.
In Vitro Phosphorylation and Inactivation of PDH by TPKI/GSK-3β. Because phosphorylation is suggested to regulate PDH activity (30), we examined the effects of TPKI/GSK-3β-catalyzed phosphorylation. As shown in Fig. 1A, α subunit of PDH included in PDH complex as well as the purified α subunit of PDH was phosphorylated by TPKI/GSK-3β in an ATP-dependent manner. The phosphorylation resulted in concomitant inactivation of PDH activity (Fig. 1B). These results confirmed the interaction of PDH with TPKI/GSK-3β. Another tau kinase, TPKII (11, 31), had no effect on PDH activity (Fig. 1B).

Localization of TPKI/GSK-3β in Mitochondria. PDH complex is located in mitochondria. We recently found that only a single GSK-3 isoform, TPKI/GSK-3β, was present in mitochondria in cerebellum neurons, but GSK-3α was not (22). This isoform-specific mitochondrial localization was observed in hippocampal neurons; 8% of TPKI/GSK-3β in the initial homogenate was recovered by subcellular fractionation in the mitochondrial pellet (Fig. 2A), in which PDH activity was predominantly recovered, but GSK-3α was not detected in this fraction (data not shown). Immunocytochemical analyses confirmed the presence of TPKI/GSK-3β in mitochondria (Fig. 2B). The specificity of the procedures was checked by substitution of normal rabbit serum or γ-immunoglobulin for the primary antibody. Thus, TPKI/GSK-3β, but not GSK-3α, is present in mitochondria and could have access to PDH.

PDH Inactivation by βA-Induced TPKI/GSK-3β in Hippocampal Neuronal Cultures. Recently PDH kinase was cloned from a rat heart cDNA library (32), but the signal cascades that lead to this kinase are unknown. TPKI/GSK-3β clearly differs from this PDH kinase, which lacks the general motif of eukaryotic Ser/Thr kinases and is similar to bacterial histidine kinases (32). To test whether TPKI/GSK-3β acts as an in vitro PDH kinase, we examined the effect of βA on PDH activity using primary cultures of rat hippocampal neurons. As shown in Fig. 3, TPKI/GSK-3β activity increased rapidly on βA treatment, reaching its maximum at 6 hr after βA exposure, and PDH was inactivated in reverse proportion to the βA-induced TPKI/GSK-3β activation. When TPKI/GSK-3β synthesis was blocked by antisense oligonucleotides (9), PDH activity showed a 2- to 3-fold increase, which corresponds with the fact that antisense oligonucleotides reduce the amount of TPKI/GSK-3β by 40% as compared to that in untreated cells (9). Thus, the inactivation of PDH activity showed a good correlation with TPKI/GSK-3β activity.

βA-Induced Impairment of ACh Synthesis in Septum Neuronal Cultures. The reduced activity of PDH may also decrease the formation of acetyl CoA for ACh production. To examine the relationship between the PDH inactivation and...
Fig. 3. Effect of βA on PDH and TPKI/GSK-3β activities in hippocampal neuronal cultures. After treatment with aggregated βA(25–35) (20 μM) (9), PDH and TPKI/GSK-3β activities were assayed as described. PDH activities were standardized against the activity of purified porcine heart PDH complex assayed simultaneously. Values are means for three determinations ± SEMs.

ACh synthesis, we investigated the cellular ACh level in primary cultures of rat septum after βA exposure. As shown in Fig. 4A, the ACh level in the culture declined to 14% relative to the control after βA (1 μM) treatment for 24 hr. Under this condition, βA (1 μM) did not affect the ChAT activity in cultured rat septum (Fig. 4B), indicating that the decreased level of acetyl CoA induced via inactivation of PDH may reduce ACh synthesis. These results suggest that TPKI/GSK-3β affects ACh synthesis through regulation of PDH activity in cholinergic neurons.

**DISCUSSION**

PDH complex, which converts pyruvate into acetyl CoA, catalyzes an irreversible reaction at the junction of the glycolytic pathway with the mitochondrial tricarboxylic acid cycle. Therefore, PDH complex is essential both for the conversion of glucose into energy and for ACh synthesis via acetyl-CoA formation in cholinergic neurons. PDH complex is composed of three catalytically active mitochondrial enzymes. We identified one component of the PDH complex, PDH, as a potential substrate for TPKI/GSK-3β by using a yeast two-hybrid system. In vitro phosphorylation of PDH by TPKI/GSK-3β resulted in inactivation of the PDH. We found that both PDH and TPKI/GSK-3β were present in mitochondria. Moreover, inactivation of PDH activity correlated well with TPKI/GSK-3β activity in primary neuronal cultures. βA treatment of the culture resulted in a 2- to 3-fold increase of phosphorylation of 43-kDa mitochondrial protein, which probably corresponds to α subunit of porcine heart PDH complex (data not shown). Because the proportion of PDH in the active form is reported to be 30% in rat brain (33), a 2- to 3-fold increase in PDH phosphorylation by βA treatment might be enough to cause the PDH inactivation seen in Fig. 3. These results suggest that TPKI/GSK-3β functions as an in vivo PDH regulator. Recently, we found that another isoform, GSK-3α, is not present in mitochondria (22). Thus, regulation of PDH is a TPKI/GSK-3β-specific function in mitochondria. In many other tissues, including liver, heart, and kidney, TPKI/GSK-3β was localized predominantly in mitochondria as determined by immunoelectron microscopy (data not shown). This result suggests that PDH regulation by TPKI/GSK-3β is a general physiological phenomenon.

In AD brain, βA deposition is a primary cause of AD pathogenesis (neuritic plaques, as well as neuronal and synaptic loss) (1, 6–8, 34). From postmortem studies, PDH activity was found to be decreased in AD brain (35–37). This decrease in PDH activity would induce the disturbed glucose metabolism (38, 39) and impaired ACh synthesis (40–43) in AD brain. Our present results provide a link between βA deposition and the PDH inactivation: βA accumulated in AD brain activates TPKI/GSK-3β, and the abnormally activated TPKI/GSK-3β phosphorylates and inactivates PDH, which leads to disturbed glucose metabolism and impaired ACh synthesis in cholinergic neurons. Injection of βA into the medial septum of rats was reported to decrease ACh release from hippocampus in vivo without affecting ChAT activity (44). A close relationship between changes in cortical cholinergic innervation and the development of neuritic plaque has also been reported (45). These observations are consistent with our proposed mechanism.

In the light of our present results, TPKI/GSK-3β occupies a critical position in the signal cascades of βA-induced neuronal death because in cytoplasm it catalyzes abnormal phosphorylation of tau and induces microtubule disruption, and in mitochondria it phosphorylates PDH, leading to mitochondrial dysfunction and reduced ACh synthesis. Thus, TPKI/GSK-3β triggers multiple neuronal death cascades and appears to play a primary role in βA neurotoxicity as observed in hippocampal neuronal cultures (9). Here, we propose the following hypothesis for AD pathogenesis. βA interacts with neurons and activates TPKI/GSK-3β. The activated TPKI/GSK-3β leads to extensive phosphorylation of tau protein and destabilization of microtubules, resulting in impaired axonal transport and neuronal death. On the other hand, the activated TPKI/GSK-3β in mitochondria phosphorylates and inactivates PDH, resulting in dysfunction of glucose metabolism, which contributes to neuronal death through energy failure. In cholinergic neurons, the inactivation of PDH causes a reduction of ACh synthesis, which may impair ACh release and synapse-forming ability. The exact sequence of the events remains to be established, and further analyses are still needed to see whether other substrates for TPKI/GSK-3β exist. Our results may provide a basis for understanding the pathogenesis of AD.