Hyperphosphorylation of Tau Induced by Naturally Secreted Amyloid-β at Nanomolar Concentrations Is Modulated by Insulin-dependent Akt-GSK3β Signaling Pathway*

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Background: Little is known about the underlying mechanisms by which extracellular amyloid-β (Aβ) induces Tau phosphorylation in Alzheimer disease (AD).

Results: Naturally secreted Aβ induced hyperphosphorylation of Tau and impaired insulin signal transduction.

Conclusion: A disturbed insulin signaling cascade may be implicated in the pathway of Aβ-induced Tau hyperphosphorylation.

Significance: These findings may explain the molecular link between Alzheimer disease and insulin signaling.

Alzheimer disease (AD) is neuropathologically characterized by the formation of senile plaques from amyloid-β (Aβ) and neurofibrillary tangles composed of phosphorylated Tau. Although there is growing evidence for the pathogenic role of soluble Aβ species in AD, the major question of how Aβ induces hyperphosphorylation of Tau remains unanswered. To address this question, we here developed a novel cell coculture system to assess the effect of extracellular Aβ at physiologically relevant levels naturally secreted from donor cells on the phosphorylation of Tau in recipient cells. Using this assay, we demonstrated that physiologically relevant levels of secreted Aβ are sufficient to cause hyperphosphorylation of Tau in recipient N2a cells expressing human Tau and in primary culture neurons. This hyperphosphorylation of Tau is inhibited by blocking Aβ production in donor cells. The expression of familial AD-linked PSEN1 mutants and APP ΔE693 mutant that induce the production of oligomeric Aβ in donor cells results in a similar hyperphosphorylation of Tau in recipient cells. The mechanism underlying the Aβ-induced Tau hyperphosphorylation is mediated by the impaired insulin signal transduction because we demonstrated that the phosphorylation of Akt and GSK3β upon insulin stimulation is less activated under this condition. Treating cells with the insulin-sensitizing drug rosiglitazone, a peroxisome proliferator-activated receptor γ agonist, attenuates the Aβ-dependent hyperphosphorylation of Tau. These findings suggest that the disturbed insulin signaling cascade may be implicated in the pathways through which soluble Aβ induces Tau phosphorylation and further support the notion that correcting insulin signal dysregulation in AD may offer a potential therapeutic approach.

Alzheimer disease (AD) is the most prevalent age-related dementing illness in humans, which is clinically characterized by memory loss and other intellectual disabilities serious enough to interfere with daily life. AD is neuropathologically characterized by the loss of neurons and synapses as well as the presence of senile plaques and neurofibrillary tangles. Senile plaques are extracellular deposits mainly composed of small peptides called amyloid-β (Aβ) that are proteolytically generated from the amyloid precursor protein (APP). Hyperphosphorylated Tau is the principal component of paired helical filaments in intracellular inclusions (i.e. neurofibrillary tangles). It is widely recognized that Aβ deposition occurs prior to the accumulation of hyperphosphorylated Tau in the AD brain. The monomer Aβ can assemble into soluble oligomers, protofibrils, and fibrils to eventually form insoluble amyloid plaques (1, 2). A growing body of evidence suggests that nonfibrillar soluble Aβ species, including oligomers, may facilitate dendritic spine loss, altered hippocampal synaptic plasticity, and impaired memory (3–5). Recent studies have suggested that soluble Aβ oligomers prepared from synthetic Aβ peptides and isolated from brain extract from AD patients accelerate Tau phosphorylation (6–8). Despite these observations indicating the pathophysiological roles of soluble Aβ species in AD pathogenesis, how Aβ induces the hyperphosphorylation of Tau in AD brains remains a fundamental and unanswered question.

Recent epidemiological studies have suggested a link between AD and type 2 diabetes mellitus (T2DM) associated...
with insulin resistance (9, 10). However, little is known about the molecular mechanisms underlying this association. Insulin/insulin receptor signaling normally plays a pivotal role in the regulation of peripheral glucose homeostasis (11). In the brain, insulin receptors are abundantly distributed in synaptic membranes of the brain in distinct patterns, with higher concentrations in the olfactory bulb, cerebral cortex, hypothalamus, cerebellum, and choroid plexus. They play a role in regulating synaptic activities that are required for learning and memory independent of glucoregulatory functions (12, 13). One possible mechanism relevant to the molecular link between T2DM and AD is an alteration of insulin signaling (14). Insulin receptors are tyrosine kinases, which autophosphorylate after their activation by binding to insulin, resulting in recognition by insulin receptor substrate adaptors. Insulin receptor substrate is then phosphorylated at tyrosines, permitting recruitment of Src homology 2 domain-containing proteins, including phosphatidylinositol 3-kinase (PI3K). Activated PI3K induces the activation of Akt, which phosphorylates various biological substrates, including glycogen synthase kinase 3β (GSK3β). It has been demonstrated that GSK3β regulates the phosphorylation of Tau (15), the main component of neurofibrillary tangles. Impairment of insulin signaling may result in a high activity of GSK3β that leads to an enhanced Tau phosphorylation, a critical event in AD pathogenesis.

With these as a background, we considered the possibility that the insulin signal pathway could be involved in the Aβ-induced hyperphosphorylation of Tau. To test this hypothesis, we established a novel cell coculture assay for the examination of the effect of naturally secreted Aβ at physiologically relevant levels on the phosphorylation of Tau.

**EXPERIMENTAL PROCEDURES**

**Constructs**—Human Tau cDNA was isolated by reverse transcription PCR using adult human brain mRNA as a template. Several isoforms of human Tau have been cloned; in this study, we chose to use the cDNA encoding the 4R1N isoform of human Tau. The cDNAs encoding the human wild-type (WT) APP and the Swedish variant APPswe in the pCB6 vector, PSEN1 L166P and ΔT440 in the pAG3 vector, and the human insulin receptor in the pcDNA3.1 vector were described previously (16–18). APP ΔE693 cDNA containing the Osaka mutation (19) was generated using PCR-based mutagenesis techniques. The resulting cDNA constructs were verified by sequence analysis.

**Antibodies**—Full-length APP and the APP C-terminal fragment were detected using a rabbit anti-APP C-terminal fragment antibody (Sigma-Aldrich). Aβ40/42 was detected using the monoclonal antibody 82E1 (IBL). The AT8 antibody (Thermo Scientific) that is raised against the phosphorylated Ser-202/Thr-205 epitope and immunoreacts with PHF-Tau in the AD brain was used to detect phosphorylated Tau. The AT180 (Thermo Scientific) and PHF6 (Santa Cruz Biotechnology, Inc.) antibodies that recognize Tau phosphorylated at Thr-231/Ser-235 and Thr-231, respectively, were also used. The Tau-1 antibody (Millipore), which specifically recognizes the dephosphorylated form of an epitope that encompasses Ser-199 and Ser-202 was used for the detection of dephosphorylated Tau. Total Tau was detected using the Tau-5 antibody (Abcam). Phosphospecific antibodies against phosphorylated Akt at serine 473 and phosphorylated GSK3β at serine 9 were used for the examination of insulin-dependent signaling (Cell Signaling Technology). The insulin receptor derivatives were detected using CT-3 (NeoMarkers), a monoclonal antibody that recognizes the C-terminal amino acid residues of human insulin receptor.

**Cell Culture and Transfection**—Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS).
Mouse neuroblastoma Neuro2a (N2a) cells were maintained in a 1:1 mixture of DMEM and Opti-MEM supplemented with 5% FBS. Primary neuronal cultures were prepared from the neocortices of embryonic day 17 rat embryos. Briefly, cortical fragments were dissociated into single cells in a dissociation solution (Sumitomo Bakelite) and resuspended in nerve culture medium (Sumitomo Bakelite). Neurons were plated onto poly-D-lysine-coated 6-well dishes. The cells were transiently transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen). To generate stable cell lines, N2a cells transfected with 2 μg of cDNA encoding human Tau (4R1N) in the pcDNA3.1 vector were selected using 1000 μg/ml G418 and screened for the expression of the transgene by Western blot analysis.

Coculture System—The BD Falcon cell culture insert system containing polyethylene terephthalate membranes with 1-μm pores (BD Biosciences) was utilized in the assay. HEK293 cells

![Figure 2. Tau phosphorylation in N2a cells cocultured with donor cells secreting Aβ.](image)

A. donor HEK293 cells were mock-transfected or transiently transfected with APP WT or the APPswe mutant construct. The levels of Aβ secreted in the medium of the donor cells were analyzed using the anti-Aβ antibody 82E1. The level of phosphorylated Tau (p-tau) detected using the AT8 and AT180 antibodies significantly increased in the N2a cells cocultured with the donor cells expressing APPswe that generated a high level of Aβ. The levels of the dephosphorylated form of Tau detected using the Tau-1 antibody were comparable among the three types of donor cell. β-Actin was visualized for normalization of loading controls. B, the results of semiquantitative analysis of phosphorylated/total Tau by densitometry are shown as mean ± S.E. (error bars) (n = 4). *, p < 0.05; **, p < 0.01 by Tukey’s test versus mock after ANOVA. C, donor HEK293 cells stably expressing WT PSEN1 or the D385A variant were transiently transfected with cDNA encoding APPswe. Note that hyperphosphorylation of Tau was markedly attenuated when the recipient cells were cocultured with the donor cells expressing PSEN1 D385A, which inhibits Aβ production. D, results of semiquantitative analysis of AT8/Tau-5 (total Tau) are shown (n = 3). **, p < 0.01 by Student’s t test.
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FIGURE 3. Hyperphosphorylation of Tau by coculture of N2a cells with cells expressing PSEN1 mutants. A, donor HEK293 cells stably expressing WT PSEN1 or FAD-linked L166P and ΔT440 mutants were transiently transfected with cDNA encoding APP WT. The level of Aβ42 increased in the medium of the donor cells that stably expressed the L166P and ΔT440 mutants. Detergent-extracted lysates of N2a cells were evaluated using the indicated antibodies. Note that the hyperphosphorylation of Tau (p-tau) and accumulation of total Tau were observed in N2a cells cocultured with the donor cells stably expressing the PSEN1 mutants (L166P and ΔT440) that produced a high level of Aβ42. B, results of semiquantitative analysis of AT8/Tau-5 (total Tau) are shown (n = 4). *, p < 0.05 by Tukey’s test versus WT PSEN1 after ANOVA. Error bars, S.E.

(donor cells) with ~90% confluence were transiently transfected with the various cDNA constructs. After 24 h, N2a cells stably expressing human Tau (4R1N) or primary rat neurons in a 6-well cell culture plate were cocultured with the host cells and incubated in a serum-free medium at 37 °C for 48 h. After incubation, the cells were subjected to biochemical analysis.

Western Blot Analysis—Cells were solubilized using a lysis buffer containing radioimmune precipitation assay buffer supplemented with 1 mM sodium orthovanadate, 5 mM sodium fluoride, and a protease inhibitor mixture (Sigma-Aldrich). The protein concentration of detergent lysates was determined using a bicinchoninic acid protein assay kit (Pierce). Cell lysates were subjected to Tris-glycine SDS-PAGE, and separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore) before incubation with appropriate antibodies. For detection of secreted Aβ, Tris-Tricine and Bicine-urea gel systems were utilized as reported previously (16). Briefly, the conditioned medium was incubated in microplate wells coated with the 82E1 antibody. Bound Aβ species were detected using the same antibody labeled with biotin, followed by development with horseradish peroxidase (HRP)-conjugated streptavidin. The color intensity of the wells reflects the amount of molecules bound to the 82E1 antibody with two or more epitopes. Molarity was calculated as a relative value standardized for dimers of Aβ.

Statistical Analyses—Data are shown as means ± S.E. The comparison between two groups was carried out by the two-tailed t test. For comparison between several groups, we used one-way analysis of variance followed by the Tukey post hoc multiple comparison test. The statistical significance was defined as a p value of 0.05 or smaller. Statistical analyses were performed using SPSS version 12.0J software (SPSS Japan).

RESULTS

Coculture System Using Dual Chamber Dishes—We attempted to establish a new coculture system that enabled the examination of whether extracellular Aβ species naturally secreted from cultured cells have priming effects on Tau phosphorylation in other populations of cultured cells. Two types of cultured cell population, donor and recipient cells, were cultured separately on a polyethylene terephthalate membrane
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FIGURE 4. Hyperphosphorylation of Tau induced by coculture of N2a cells with cells expressing APP oligomer mutation. A, donor HEK293 cells were transiently transfected with WT APP or the ΔE693 mutant, which induced the production of oligomer Aβ. Detergent-extracted lysates of N2a cells were analyzed using the indicated antibodies. The hyperphosphorylation of Tau (p-tau) and accumulation of total Tau were observed in the N2a cells cocultured with the donor cells transfected with the APP ΔE693 mutant. B, results of semiquantitative analysis of AT8/Tau-5 (total Tau) are shown (n = 4). *, p < 0.05 by Student’s t test. Error bars, S.E.

with 1-μm pores using a dual chamber. Naturally secreted Aβ peptides can induce biological activities in recipient cells following their transport across the polyethylene terephthalate membrane (Fig. 1A). N2a cells stably transfected with human 4R1N Tau were used as the recipient cells, in which exogenously expressed human Tau was easily visualized using the anti-Tau antibodies (Fig. 1B). HEK293 donor cells were transiently transfected with various cDNA constructs to secrete Aβ. When the donor HEK293 cells were transfected with the APP Swedish mutant (APPSwe), the level of Aβ in coculture medium determined by SDS-PAGE was comparable with that found in cerebrospinal fluid of AD patients (Fig. 1C). Further quantification of Aβ40 and Aβ42 by sandwich ELISA confirmed that their levels in the medium were comparable with those in cerebrospinal fluid of AD patients (supplemental Table 1). Thus, this coculture system allows us to assess the effects of naturally secreted Aβ species at physiologically relevant levels on recipient cells.

Naturally Secreted Aβ-dependent Hyperphosphorylation of Tau—We began by asking whether Aβ peptides naturally secreted from donor cells can cause any change in Tau phosphorylation in recipient cells. The recipient N2a cells stably expressing human Tau (4R1N) were cocultured with the HEK293 donor cells that were transiently mock-transfected or transfected with cDNA encoding WT APP or the Swedish mutant. The level of phosphorylated Tau detected using the AT8 and AT180 antibodies in the recipient N2a cells cocultured with the APPswe-expressing donor cells was significantly higher than that in the recipient cells cocultured with mock-transfected donor cells (Fig. 2A and B). The levels of phosphorylated Tau in the recipient cells detected using the Tau-1 antibody were comparable among the three types of donor cell. To examine the possible effect of overproduction of soluble APP by donor cells on phosphorylation of Tau, we transiently transfected cDNA encoding soluble APPα into donor cells. Production of soluble APPα in the medium revealed no apparent alteration of the level of phosphorylated Tau in the recipient cells (supplemental Fig. 1, A and B).

We then determined whether hyperphosphorylation of Tau in the recipient cells is dependent on soluble Aβ generated by the donor cells. The increase in phosphorylated Tau level in the recipient cells was not observed when these cells were cocultured with the donor cells stably expressing the dominant negative PS1 D385A variant, which largely blocked Aβ production (Fig. 2 C and D). Similarly, treating the donor cells with the γ-secretase inhibitor DAPT significantly reduced the level of phosphorylated Tau in the recipient cells (supplemental Fig. 2, A and B). These results suggest that the hyperphosphorylation of Tau in the recipient cells is induced by Aβ naturally secreted from the donor cells at physiologically relevant levels in an Aβ-dependent manner.

Aβ Species Generated by Familial AD (FAD)-linked Mutants Enhance Phosphorylation of Tau—We next examined the effects of Aβ species secreted as induced by FAD-linked mutations of PSEN1 and APP. We chose to use the donor HEK293 cells stably expressing PSEN1 mutations of L166P and ΔT440, which were further transfected with cDNA encoding WT APP. The expression of the FAD-linked PSEN1 mutations resulted in overproduction of Aβ42 in the medium as reported previously (16, 17) (Fig. 3A). When the N2a cells were cocultured with the donor cells expressing the PSEN1 mutants, the levels of phosphorylated and total Tau were markedly higher than those detected in cells cocultured with WT PSEN1-expressing cells (Fig. 3, A and B). The levels of dephosphorylated Tau detected
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We next examined the effects of the FAD-linked APP ΔE693 mutant, which has been shown to induce the production of the oligomeric form of Aβ species (19). Cells expressing this mutant produce an Aβ variant lacking glutamate 22 (E22Δ), which was reported to readily induce the formation of oligomers but not amyloid fibrils in in vitro and in vivo models (19, 22). The donor HEK293 cells were transfected with cDNA encoding APP WT or the ΔE693 mutant, and the level of Aβ was examined by sandwich ELISA. Although the levels of Aβ(1–40) and Aβ(1–42) in the medium of cells expressing the APP ΔE693 mutant were very low as determined by conventional sandwich ELISA, as reported previously (19), oligomer/dimer-specific ELISA revealed that the levels of Aβ oligomer were much higher than those in the medium of APP WT-expressing donor cells (supplemental Table 1). The coculture of the N2a recipient cells with the donor cells expressing the APP ΔE693 mutant resulted in a significant increase in the levels of phosphorylated and total Tau (Fig. 4, A and B).

Impeded Insulin Signal Transduction in Aβ-induced Hyperphosphorylation of Tau—In an attempt to examine the possible contribution of the insulin signal cascade to Aβ-induced Tau phosphorylation, we determined the levels of phosphorylated Akt (Ser-473) and GSK3β (Ser-9), which are downstream molecules of insulin signal transduction and are immediately activated upon insulin treatment. The recipient N2a cells cocultured with mock-transfected or APPswe-expressing donor cells were treated with insulin (1 μM), and the recipient cell lysates were analyzed at the indicated time points. The phosphorylated Akt (Ser-473) and GSK3β (Ser-9) levels in the recipient cells cocultured with mock-transfected donor cells increased upon insulin stimulation in a time-dependent manner (Fig. 5, A and B). In contrast, a sufficient increase in the levels of pAkt (Ser-473) and pGSK3β (Ser-9) upon insulin stimulation was not observed in the N2a cells cocultured with APPswe-expressing donor cells that produce high levels of Aβ (Fig. 5, A and B).

Hyperphosphorylation of Endogenous Tau in Primary Neurons by Various Aβ Species—We further investigated the effects of Aβ naturally secreted from the donor cells on the recipient cells using primary neurons prepared from rat embryos. Similar to the results obtained from the experiments using N2a cells, the level of phosphorylated endogenous Tau in primary neurons detected using the AT8 and AT180 antibodies significantly increased when the neurons were cocultured with the APPswe-expressing donor cells (Fig. 6, A and B). The mobility of endogenous total Tau from primary neurons was altered by the coculture with the donor cells stably expressing PS1 D385A variant to inhibit Aβ production (supplemental Fig. 3, A and B).

We next examined the effects of Aβ species whose production was induced by the FAD-linked PSEN1 mutants (L166P and ΔT440) and APP ΔE693 mutant on the phosphorylation of endogenous Tau in primary neurons. The level of phosphorylated endogenous Tau increased in the primary neurons cocultured with the donor cells expressing the PSEN1 mutants (L166P and ΔT440) (Fig. 7, A and B). Similarly, the coculture of primary neurons with the donor cells producing oligomeric Aβ species, as induced by the expression of the APP ΔE693 mutant, resulted in a significant increase in the level of phosphorylated Tau in the primary neurons (Fig. 7, C and D). The level of dephosphorylated Tau was not apparently altered by the expression of the FAD-linked mutants in donor cells.

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Impaired Insulin Signaling and Effects of Insulin Signal Sensitizer Drugs on Primary Neurons—We next treated neurons with insulin to determine whether insulin signal transduction is impaired in primary neurons cocultured with donor cells expressing APPswe. Insulin-dependent phosphorylations of Akt (Ser-473) and GSK3β (Ser-9) were observed in the primary neurons cocultured with the mock-transfected donor cells. In contrast, phosphorylations of Akt (Ser-473) and GSK3β (Ser-9) were insufficiently activated upon insulin treatment of primary neurons cocultured with APPswe-expressing cells (Figs. 8, A and B).

Because these results raise the possibility that the insulin signal cascade mediates the Aβ-induced hyperphosphorylation of Tau, we then examined whether improving insulin signaling might have beneficial effects on Tau phosphorylation. To test this hypothesis, we attempted to enhance insulin signal transduction by transient transfection of cDNA encoding the insulin receptor into the recipient cells. Overexpression of the insulin receptor in the presence of insulin (100 nM) in the recipient cells significantly decreased the level of phosphorylated Tau induced by Aβ (Fig. 9, A and B). We next examined the insulin-sensitizing drugs pioglitazone and rosiglitazone, both of which are used to ameliorate insulin resistance in patients with T2D. Treating the primary culture neurons with pioglitazone (50 μM) or rosiglitazone (50 μM) in the presence of insulin (100 nM) attenuated the phosphorylation of endogenous Tau (supplemental Fig. 4, A and B). Treatment with rosiglitazone decreased the level of phosphorylated Tau more markedly than that with pioglitazone. We then examined the effect of rosiglitazone on the phosphorylation of endogenous Tau in the primary neurons cocultured with the donor cells in which the Aβ-induced hyperphosphorylation of Tau occurs. When the primary neurons cocultured with the APPswe-expressing cells were treated with rosiglitazone (50 μM), the level of hyperphosphorylated endogenous Tau markedly decreased (Fig. 9, C and D) without changing the level of Aβ in the medium (supplemental Fig. 4C). The levels of phosphorylated Tau were comparable among the three types of donor cell (Fig. 9C).

DISCUSSION

Much interest has been focused on the clarification of the molecular mechanisms by which extracellular Aβ induces the hyperphosphorylation of Tau in the pathogenic pathway of AD that may be amenable to pharmacological intervention before significant neurodegeneration has occurred. Here, using a new cell coculture assay, we found the following: 1) naturally secreted Aβ at physiologically relevant levels is sufficient to induce the hyperphosphorylation of endogenous and exoge-
nous Tau; 2) the insulin-dependent Akt-GSK3β signal pathway is insufficiently activated under the conditions where extracellular Aβ-induced Tau hyperphosphorylation occurs; and 3) treatment with the insulin-sensitizing drug rosiglitazone protected against the extracellular Aβ-induced hyperphosphorylation of Tau. These findings may provide new insights into the potential mechanisms underlying AD pathophysiology that link Aβ-induced Tau phosphorylation with insulin signal transduction disturbance.

The coculture system used in this study has several advantages for exploring Aβ neurotoxicity occurring in the AD brain. Cell-derived Aβ species are distinct from the widely used synthetic Aβ preparations as follows. First, the Aβ produced by donor cells is naturally generated from human full-length APP

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**FIGURE 7.** *Hyperphosphorylation of endogenous Tau in primary neurons cocultured with cells expressing FAD-linked mutants.* A, rat primary neurons were cocultured with donor cells stably expressing PSEN1 L166P and ΔT440, which were further transiently transfected with cDNA encoding WT APP. Detergent-extracted lysates of the primary neurons were evaluated using the indicated antibodies. The hyperphosphorylation of endogenous Tau (p-tau) was observed in the primary neurons cocultured with the donor cells stably expressing PSEN1 mutants. B, results of semiquantitative analysis of AT8/Tau-5 (total Tau) in the recipient primary neurons are shown (*n* = 4). *, *p* < 0.05 by Tukey’s test versus WT after ANOVA. C, primary neurons were cocultured with donor cells transiently transfected with cDNA encoding WT APP or the ΔE693 mutant, which induced the production of the oligomeric form of Aβ species. Detergent-extracted lysates of the recipient primary neurons were analyzed using the indicated antibodies. The phosphorylation of endogenous Tau was significantly enhanced in the primary neurons cocultured with the donor cells expressing the APP ΔE693 mutant. D, results of semiquantitative analysis of AT8/Tau-5 (total Tau) in the recipient primary neurons are shown (*n* = 4). *, *p* < 0.05 by Student’s *t* test. Error bars, S.E.
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by proteolytic processing and is composed of both Aβ40 and Aβ42 species similar to those that occur in the brain. In contrast, synthetic Aβ peptides have a single defined length, usually Aβ40 or Aβ42. Furthermore, synthetic Aβ peptides require several steps of chemical preparation and incubation for the peptides to form specific conformations, which may cause propensity alterations that are distinct from those in the naturally generated Aβ species. Second, naturally secreted Aβ species have biological effects at low nanomolar concentrations similar to those in the human brain and cerebrospinal fluid, as shown in this study, whereas synthetic Aβ peptides are typically required at micromolar concentrations to obtain similar biological activities (supplemental Fig. 5). The naturally generated Aβ species at physiological concentrations show much higher biological activities than the synthetic Aβ peptides. In addition, our system enables the evaluation of the continuous effects of secreted Aβ on the recipient cells during the course of their coculture with the donor cells.

Recent epidemiological studies, such as the Rotterdam and Hisayama studies, have provided evidence that the insulin resistance associated with T2DM is a strong risk factor for AD (23, 24). The underlying mechanisms linking the development of insulin resistance with AD are not fully understood. Several lines of evidence suggest that insulin regulates the phosphorylation of Tau in neuronal cells (25, 26). Furthermore, insulin receptor substrate 2 knock-out mice showed an enhanced deposition of hyperphosphorylated Tau in hippocampal neurons (27). Recently, Takeda et al. (28) have reported that reduced insulin levels in brains as well as enhanced neuronal insulin resistance are observed in AD transgenic mice crossed with ob/ob diabetic mice, which developed a more severe cognitive impairment. Supporting these findings obtained from animal models, data from a biochemical study of autopsied brains also suggest the presence of insulin resistance in the brains of AD patients. A recent study has suggested that insulin-P13K-Akt signaling is attenuated in the brains of AD patients (29), particularly in those of AD patients with the complication of T2DM (30). Taken together, these results suggest that insulin receptor-mediated signaling disturbance may be a mechanistic link between insulin resistance in the brain and AD pathophysiology. In this regard, our study raises an intriguing issue that the insulin-mediated signaling pathway can directly result from extracellular Aβ toxicity, eventually leading to neuronal dysfunction caused by the hyperphosphorylation of Tau.

Although our findings may provide new insights into AD pathogenesis, the mechanism(s) by which soluble Aβ impairs the insulin receptor-mediated signaling pathway is not precisely clarified. Previous studies have also shown that the reduced responsiveness of insulin receptors and attenuated downstream signaling for insulin stimulation are caused by Aβ oligomers (31–34). It has been shown that the down-regulation of insulin receptors on the cell surface is induced by Aβ-derived diffusible ligands acting as specific pathogenic ligands binding to particular synapses (34). However, our experiment using a cell surface biotinylation assay revealed that the cell surface localization of insulin receptors in primary neurons did not change under the condition where impaired insulin signal transduction occurred due to the coculture of the primary neurons with the Aβ-producing donor cells (data not shown). Further studies will be required to determine the primary effects of extracellular Aβ on the function and localization of insulin receptors. Moreover, Tau has been found to be phosphorylated at over 30 serine/threonine residues in the brains of AD patients, and approximately one-half of these are canonical sites for proline-directed protein kinases, including MAPK, cyclin-dependent kinase, and GSK3β (35–37). Recently, cultured neurons differentiated from induced pluripotent stem cells derived from primary fibroblasts of FAD patients with APP

FIGURE 8. Insulin-dependent phosphorylation of Akt (Ser-473) and GSK3β (Ser-9) in recipient primary neurons. A, rat primary neurons cocultured with donor cells mock-transfected or transiently transfected with cDNA encoding APPswe were stimulated with insulin (1 μM) to induce insulin signal cascade activation. Following the synchronized stimulation, the cell lysates of the primary neurons were collected at the indicated time points. Insulin-dependent phosphorylation of pAkt (Ser-473) and GSK3β (Ser-9) was examined by immunoblot analysis using phosphospecific antibodies. The activation of pAkt and pGSK3β upon insulin stimulation was insufficient in the primary neurons cocultured with the donor cells expressing the APPswe mutant. B, results of quantification of pAkt and pGSK3β normalized by their total amount are shown as fold changes over the baseline. The solid line indicates values obtained from coculture with mock-transfected cells. The dotted line indicates values from coculture with APPswe-expressing cells. Results of three independent experiments are shown. *, p < 0.05 by Student’s t test. Error bars, S.E.
duplication have been reported to show significantly higher levels of active GSK3β and phosphorylated Tau (Thr-231) (38). The present study also showed that the inefficient activation of Akt-GSK3β signaling is relevant to Aβ-induced Tau phosphorylation. However, it is conceivable that different pathways function in concert in Tau phosphorylation in the AD brain.

Understanding the effects of Aβ on the hyperphosphorylation of Tau mediated by the insulin signal pathway may provide a new potential therapeutic approach. In this study, we showed that the insulin-sensitizing drug rosiglitazone has a protective effect against Aβ-induced hyperphosphorylation of Tau in primary neurons. A plausible mechanism underlying this protection is provided by our demonstration that rosiglitazone potentiates insulin signal transduction (data not shown), although other pleiotropic mechanisms of action exerted by rosiglitazone are also possible. The rationale for using rosiglitazone for the treatment of brain diseases is supported by the findings that peroxisome proliferator-activated receptor γ is expressed in the brain regions, including the hippocampus (39). Furthermore, rosiglitazone can penetrate the blood-brain barrier (40). In the mouse AD model, Pedersen et al. (41) reported that rosiglitazone attenuated deficits in learning and memory without changing Aβ deposition. Previous human clinical trials exploring the potential effectiveness of peroxisome proliferator-activated receptor γ agonists, including rosiglitazone and pioglitazone, for AD patients showed contradictory results regarding the efficacy of these drugs. The results of Watson et al. (42) demonstrated a positive correlation between insulin level and cognitive improvement in patients treated with rosiglitazone compared with those treated with placebo. Gold

FIGURE 9. Protective effect due to enhanced insulin signal transduction against endogenous Tau phosphorylation. A, the recipient N2a cells with or without transient transfection of cDNA encoding the insulin receptor were cocultured with donor cells expressing APPswe. Detergent-extracted lysates of the recipient cells were analyzed using the indicated antibodies. Expression of insulin receptor in the presence of insulin reduced the level of phosphorylated Tau (p-tau) in the recipient cells. B, results of semiquantitative analysis of AT8/Tau-5 (total Tau) in the recipient Na2 cells are shown (n = 4). *, p < 0.05; **, p < 0.01 Tukey’s test versus WT after ANOVA. C, rat primary neurons cocultured with donor cells transiently transfected with cDNA encoding APPswe were treated with vehicle only or with rosiglitazone (50 μM) in the presence of insulin (100 nm). Detergent-extracted lysates of the recipient cells were analyzed using the indicated antibodies. Treatment with rosiglitazone showed a protective effect against the hyperphosphorylation of Tau induced by Aβ secreted at physiologically relevant levels. D, results of semiquantitative analysis of AT8/Tau-5 (total Tau) in the recipient primary neurons are shown (n = 3). *, p < 0.05 by Tukey’s test versus WT after ANOVA. Error bars, S.E.
et al. (43) have recently conducted a multicenter randomized double-blind clinical study, which revealed no evidence of efficacy of rosiglitazone for improving cognitive function in AD patients. In contrast, Sato et al. (44) reported the significant improvements of cognition and regional blood flow in patients with AD complicated by T2DM treated with pioglitazone compared with those treated with placebo controls. A recent clinical trial showed that the administration of intranasal insulin improved cognitive performance in elderly patients with early AD and amnestic mild cognitive impairment (45). Although the precise mechanisms underlying these effects of therapies targeting brain insulin signaling have not yet been clarified, correcting insulin signal dysregulation in the CNS may be a potential therapeutic approach for some AD patients, particularly when AD is a consequence of insulin dysregulation or the AD patients have the T2DM complication.

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