The FASEB Journal • FJ Express

Amino-truncated amyloid β-peptide (Aβ5-40/42) produced from caspase-cleaved amyloid precursor protein is deposited in Alzheimer's disease brain

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SPECIFIC AIMS

Caspase activation and apoptosis are implicated in neuronal death in Alzheimer's disease (AD). We analyzed the effects of the caspase-mediated cleavage of amyloid precursor protein (APP) on amyloid β-peptide (Aβ) production with special consideration of the generation of amino-terminally truncated Aβ.

PRINCIPAL FINDINGS

1. Evidence for altered Aβ generation from cells expressing caspase-cleaved form of APP

APP is cleaved by caspases in its cytoplasmic domain, subsequently generating APP lacking C-terminal 31 amino acids (APPΔC). Human neuroblastoma SH-SY5Y cells stably transfected with wild-type APP or APPΔC were established and designated SH-APP and SH-APPΔC cells, respectively. We selected two pairs of SH-APP and SH-APPΔC cells expressing similar APP levels (designated SH-APP-1, -2, and SH-APPΔC-1, -2 cells). SH-APP-2 and SH-APPΔC-2 expressed ~ twice as much APP as did SH-APP-1 and SH-APPΔC-1. Two types of sandwich ELISA were used to measure Aβ in conditioned media from these cells. BNT77-based ELISA detected N-terminal-intact and truncated Aβ (Aβ40total and Aβ42total), but not Aβ17-40 (p3 fragment); BAN50-based ELISA detected only N-terminal-intact Aβ (mainly Aβ1-40 and Aβ1-42). BNT77-based ELISA showed that SH-APP and SH-APPΔC cells secreted comparable levels of Aβ40total and Aβ42total. BAN50-based ELISA revealed that the amounts of Aβ1-40 and Aβ1-42 in SH-APPΔC cells were decreased to ~30% of those found in SH-APP cells. These data suggest that N-terminally truncated Aβ is increased relative to total Aβ in SH-APPΔC cells.

We next analyzed C-terminal fragments (CTF) of APP by immunoprecipitation Western blot with 4G8 antibody. Two CTF bands (~10 kDa α-CTF and ~12 kDa β-CTF) and a faint band (β′-CTF, ~11 kDa) were detected in cell lysates of SH-APP. Similarly, two bands (~6 kDa α-CTFΔC and ~8 kDa β-CTFΔC) and a faint band (β′-CTFΔC, ~7 kDa) were observed in SH-APPΔC cell samples. The relative level of β′-CTFΔC was increased in SH-APPΔC cells. Steady-state levels of β-CTFΔC and α-CTFΔC in SH-APPΔC cells were lower than those of β-CTF and α-CTF in SH-APP cells.

We compared the generation of secreted APP (sAPP) in SH-APP and SH-APPΔC cells. Immunoprecipitation Western blot showed that levels of total sAPP and sAPP-α (sAPP derived from α-secretase cleavage) were ~4-fold higher in SH-APPΔC cells, compared with SH-APP cells.

2. Increased production of amino-truncated Aβ from caspase-cleaved APP

We then analyzed altered Aβ secretion in SH-APPΔC cells using immunoprecipitation Western blot. BAN50 antibody immunoprecipitated Aβ1-40 and Aβ1-42 (band 1, comigrating with synthetic Aβ1-40/42). These immunoreactivities were decreased in media from SH-APPΔC cells, compared with SH-APP cell media. N-terminal-intact Aβ (Aβ1-40 and Aβ1-42) and the smaller fragment (band 3, most likely Aβ11-40 and Aβ11-42) were detected in media from SH-APP cells by BNT77 immunoprecipitation. In contrast, the intensities of bands 1 and 3 were reduced, and the intensity of band 2 was increased in SH-APPΔC media (**Fig. 1*A***). We did not observe any band comigrating with Aβ17-40 (p3 fragment) in the BNT77 immunoprecipitates.

To identify secreted Aβ species, BNT77 immunoprecipitates were analyzed using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS). Two major peaks of Aβ1-40 and Aβ11-40 were detected in conditioned media from

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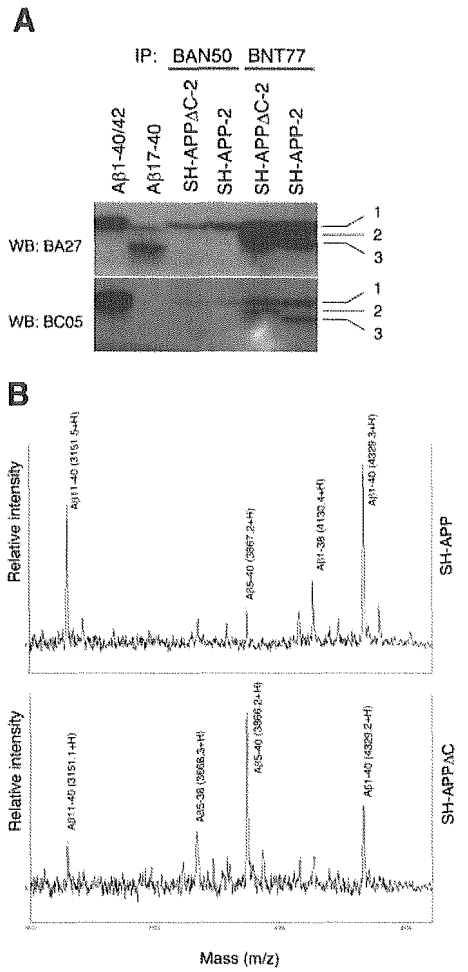

Figure 1. Immunoprecipitation Western blot and mass spectrometric analyses of secreted Aβ. *A)* Secreted Aβ peptides were immunoprecipitated with BAN50 or BNT77 antibodies and subjected to Tris-Tricine SDS-PAGE and Western blot analyses with BA27 or BC05. Synthetic Aβ1-40/42 and Aβ17-40 were simultaneously electrophoresed as markers. In BNT77 immunoprecipitates, 3 bands were detected. Band 1 corresponded to N-terminally intact Aβ1-40/42 and band 3 possibly represented Aβ11-40/42. Bands 1 and 3 were the major Aβ species in SH-APP cells. Conversely, in SH-APPΔC cells, the intensity of band 2 was markedly increased whereas that of bands 1 and 3 was reduced. In BAN50 immunoprecipitates, only band 1 was detected. *B)* Secreted Aβ was immunoprecipitated with BNT77 from conditioned media of SH-APP or SH-APPΔC cells and analyzed by MALDI-TOF-MS. Peaks were identified according to observed molecular and theoretical masses of Aβ and its variants. Aβ1-40, Aβ11-40, and some C-terminally truncated Aβ forms such as Aβ1-38 were identified in the sample from SH-APP (upper). Peak intensities of Aβ1-40 and Aβ11-40 were reduced whereas that of Aβ5-40 was markedly increased in the SH-APPΔC sample (lower). Aβ5–38 was also seen in this sample.

SH-APP cells. In contrast, the relative peak intensity of Aβ5-40 was markedly increased, whereas peak intensities of Aβ1-40 and Aβ11-40 were decreased in SH-APPΔC media (Fig. 1*B*). These data show that N-terminally truncated Aβ (starting at Arg5) is markedly increased in media from SH-APPΔC cells. Aβ11-40/42 appears to be generated through processing of APP by BACE1 and γ-secretase, as BACE1 alternatively cleaves between Tyr10-Glu11 in the Aβ sequence. Increased levels of Aβ5-40 and decreased levels of Aβ1-40 and Aβ11-40 were observed in media from HEK293 cells transiently transfected with APPΔC, compared with those transfected with APP.

We further analyzed the levels and species of intracellular Aβ in SH-APP and SH-APPΔC cells by sensitive Western blot. Aβ1-40 and Aβ1-42 levels were comparable between samples from SH-APP and SH-APPΔC cells. The intracellular Aβ1-42/Aβ1-40 ratio was ~0.4 in both cell lines. These results suggest that the generation of intracellular Aβ is unaffected by the C-terminal truncation of APP.

3. Aβ5-40/42 generation involves altered β cleavage of APP

To determine the processing mechanism by which Aβ5-40/42 is generated from APPΔC, we treated SH-APPΔC and SH-APP cells with a specific inhibitor of BACE, OM99-2. Secreted Aβ was analyzed by immunoprecipitation Western blot and mass spectrometry. In OM99-2-treated SH-APPΔC cells, Aβ1-40 secretion was significantly decreased, but the Aβ5-40 level was not altered compared with that in untreated cells. SH-APP cells treated with the inhibitor secreted significantly reduced Aβ1-40 and increased Aβ5-40 levels. These data suggest that cleavage between Phe4 and Arg5 is not mediated by BACE1. BACE1 inhibition promotes the secretion of Aβ5-40. To establish whether α-secretase-like proteases are responsible for Aβ5-40/42 generation, we incubated SH-APPΔC cells with a TACE (tumor necrosis factor-α converting enzyme) inhibitor, TAPI-1, which inhibits α-secretase. Incubation with 20 μM TAPI-1 resulted in increased Aβ1-40 and decreased Aβ5-40 secretion, suggesting that α-secretase-like proteases are involved in Aβ5-40/42 production.

4. Immunohistochemical analysis of Aβ5-40/42 in AD brain

To determine whether Aβ5-40/42 is present in human brain tissues, we generated a specific antibody to the N-terminal end region of this Aβ species (designated the Aβ5 antibody). In Western blot analyses, the Aβ5 antibody reacted with Aβ5-40, but not with Aβ1-40, whereas the BAN50 antibody recognized only Aβ1-40. The Aβ5 antibody immunostained vessels in the AD brain, indicating the deposition of Aβ5-40/42, particularly in vascular lesions with amyloid angiopathy. In nearby sections, another Aβ antibody (6E10) labeled more vessels than did the Aβ5 antibody. Amyloid angiopathy in the smaller sized vessels tended to be negative or weakly positive for Aβ5. In addition, Aβ5 antibody stained numerous neurofibrillary tangles (NFT), suggesting that Aβ5-40/42 may be deposited in the NFT. Although a small number of senile plaques were positive for Aβ5 in some cases, the staining was not as consistent as that of vessels and NFT.

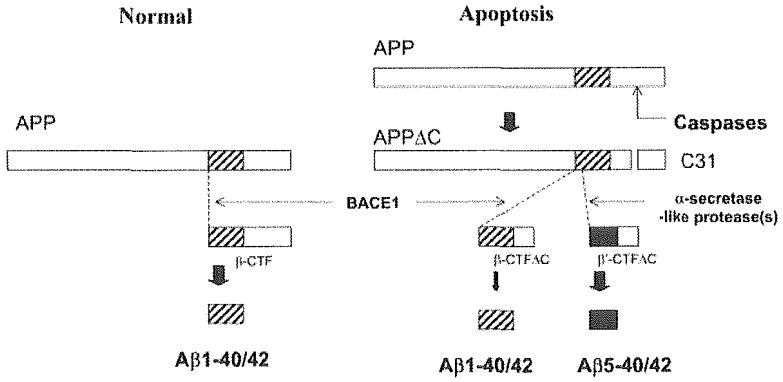

Figure 2. The possible mechanism of the generation of Aβ5-40/42. During apoptosis, APP is cleaved by caspases to form APPΔC and a C-terminal fragment consisting of 31 amino acids (C31). APPΔC is preferentially processed between Phe4 and Arg5 to generate β'-CTFΔC possibly through alternative processing by α-secretase-like protease(s). Subsequent γ-secretase cleavage results in the formation of Aβ5-40/42.

5. Cleavage at the Aβ5 site occurs when wild-type APP-expressing cells undergo apoptosis

Finally, we investigated whether APP processing to generate Aβ5-40/42 occurs during apoptosis of wild-type APP-expressing cells. SH-APP cells were exposed to MG132 or MG132 plus staurosporine. Western blots using an AB5942 antibody specific for the caspase-generated neo epitope of APP reveal that exposure to these agents leads to caspase-mediated cleavage at the cytoplasmic region of APP. We examined CTF production from caspase-processed APP by immunoprecipitation Western blot analysis with 4G8 and AB5942 antibodies. Cells treated with MG132 plus staurosporine contained significant amounts of β-CTFΔC, β'-CTFΔC, and α-CTFΔC, consistent with data from SH-APPΔC cells. The results suggest that cleavage at the Aβ5 site occurs during apoptosis in SH-APP cells.

CONCLUSIONS AND SIGNIFICANCE

Recent evidence suggests that apoptosis underlies the neuronal death seen in AD. Active forms of caspases and the caspase-cleaved APP have been detected in AD brain tissues, but it is not clear whether the caspase cleavage of APP affects Aβ formation. In this study, we have clearly demonstrated that such APP cleavage promotes the secretion of a distinct amino-truncated Aβ species (Aβ5-40/42). Our data provide the first evidentiary connection between caspase activation and the formation of amino-truncated Aβ. Our results are consistent with and expand upon data from previous studies that measured N-terminally intact Aβ, but not amino-truncated Aβ.

We used inhibitors of BACE and α-secretase to investigate the mechanism of Aβ5-40/42 generation. After treatment of SH-APP cells with a BACE inhibitor, OM99-2, Aβ1-40 levels were decreased, whereas Aβ5-40 levels were increased. Treatment of SH-APPΔC cells with TAPI-1 led to decreased Aβ5-40 and increased Aβ1-40 levels. The data strongly suggest that cleavage at the Aβ5 site is not ascribed to BACE1 activity, but mediated by α-secretase-like proteases (e.g., ADAM family proteases, including TACE and ADAM10). This is consistent with the finding that secretion of p3 (Aβ17-40), a product derived from α-secretase cleavage, is significantly increased in cells expressing APPΔC. BACE2 functions as an alternative α-secretase, but may not be involved in Aβ5-40/42 generation, since OM99-2 inhibits BACE1 and BACE2.

It has been established that wild-type APP undergoes caspase cleavage during apoptosis, so it is reasonable to assume that subsequent cleavage at the Aβ5 site occurs in apoptotic cells. We show evidence that APPΔC is generated in SH-APP cells exposed to MG-132 and staurosporine. β'-CTFΔC, which corresponds to a precursor of Aβ5-40/42, is formed in these apoptotic cells. Accordingly, we conclude that Aβ5-40/42 is generated after caspase activation (**Fig. 2**).

Our sensitive Western blot analyses indicate that the majority of intracellular Aβ consist of Aβ1-40/42 in wild-type APP- and APPΔC-expressing cells. Since two distinct pathways appear to exist for extracellular and intracellular pools of Aβ, amino-truncated Aβ peptides, including Aβ5-40/42, are likely to be produced mainly in the extracellular Aβ pathway.

Our immunohistochemical staining with the Aβ5 antibody revealed that Aβ5-40/42 species are deposited in some vessels with amyloid angiopathy in AD brain tissue. This may reflect the in vivo occurrence of caspase cleavage of APP. The observation that Aβ5 antibody labels NFT is intriguing, considering that activation of caspases is suggested to occur in neurons bearing NFT. Our data are consistent with previous reports that considerable N-terminal modifications of Aβ are seen in AD cortices and leptomeninges. Such amino-truncated Aβ species may be instrumental in the amyloidosis process.

We suggest that caspase activation in the AD brain results in the formation of APPΔC, leading to the increased production and deposition of N-terminally truncated Aβ5-40/42. Further research on the in vivo generation of this Aβ species is needed to clarify its pathological role in Aβ deposition and neuronal death in AD. FJ

Glypican-1 as an Aβ binding HSPG in the human brain: its localization in DIG domains and possible roles in the pathogenesis of Alzheimer's disease[1]

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SPECIFIC AIMS

The amyloid deposition whose major component is the 39-43 amino acid peptide, termed β-amyloid protein (Aβ), is considered to be important in Alzheimer's disease (AD) pathology, but the precise mechanism of its accumulation in AD brain is unclear. Although there are studies of the association between Aβ and heparan sulfate proteoglycans (HSPGs), they demonstrated co-localization in plaques or binding ability of HSPGs derived from tissues other than human brain. It is not fully understood whether HSPGs are involved in neuronal cell death in AD brain. Our aims are to identify Aβ binding HSPG(s) from human brain and to investigate their roles in Aβ accumulation and neuronal cell death.

PRINCIPAL FINDINGS

1. Identification of glypican-1 as an Aβ binding HSPG from human brain

To identify HSPGs derived from the human brain with the capacity to bind Aβ, control human brain lysates were separated using anion exchange DEAE-Sepharose chromatography and fractions containing possible Aβ binding protein(s) were determined by Aβ binding assay. We found that fractions containing HSPGs exclusively showed the binding activity to fibrillar Aβ in an HS chain-dependent manner. Earlier studies reported that Aβ binding to HS chains prevents heparanase-catalyzed degradation of HS chains. Thus, we examined whether preincubation of the HSPG containing DEAE fractions with Aβ alters the sensitivity of HSPGs to heparitinase treatment. Without preincubation with Aβ, several bands (~200, ~100, ~60, ~40 kDa) were detected with 3G10 mAb in the DEAE fractions, indicating they contained plural HSPGs. Preincubation with Aβ resulted in the disappearance of the ~60 kDa band; the intensity of the other bands was relatively unchanged, suggesting that an HSPG with the ~60 kDa core protein bound to Aβ preferentially and prevented heparitinase-catalyzed degradation of HS chains. Glypicans are known as HSPGs with a ~60 kDa core protein; six glypicans (glypican-1 to -6) have been cloned. Glypican-1 is the major HSPG expressed in the adult brain. We performed the same incubation experiments to clarify the identity of the ~60 kDa band using anti-glypican-1 mAb. A 60 kDa band of glypican-1 core protein was detected in the lysates; preincubation of the lysates with Aβ resulted in a marked decrease of this 60 kDa band and the appearance of a smear band (>100 kDa) probably representing intact glypican-1. Pretreatment of lysates with heparitinase before incubation with Aβ recovered the 60 kDa band, suggesting that Aβ was unable to bind to heparitinase-treated glypican-1. These results suggest that glypican-1 derived from the human brain can bind to Aβ in an HS chain-dependent manner.

2. Aβ binding to glypican-1 depends on its aggregation state

It was reported that heparin or mouse EHS HSPG binds fibrillar Aβ (fAβ) but not non-fibrillar Aβ (non-fAβ) with high affinity. In the present in vitro analysis of Aβ binding to glypican-1, the core protein of glypican-1 could be detected after incubation with non-fibrillar Aβ but not after with fibrillar Aβ at levels similar to those observed in the untreated sample, suggesting that non-fibrillar Aβ had little or no binding ability to glypican-1. Binding of non-fibrillar Aβ to HSPGs was not observed on dot blot membranes.

3. Glypican-1 is a major HSPG in DIG domains from human brains

Glypican-1 is a GPI-anchored HSPG; most, if not all, GPI-anchored proteins are localized in special mem-

[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.03-1040fje; doi: 10.1096/fj.03-1040fje

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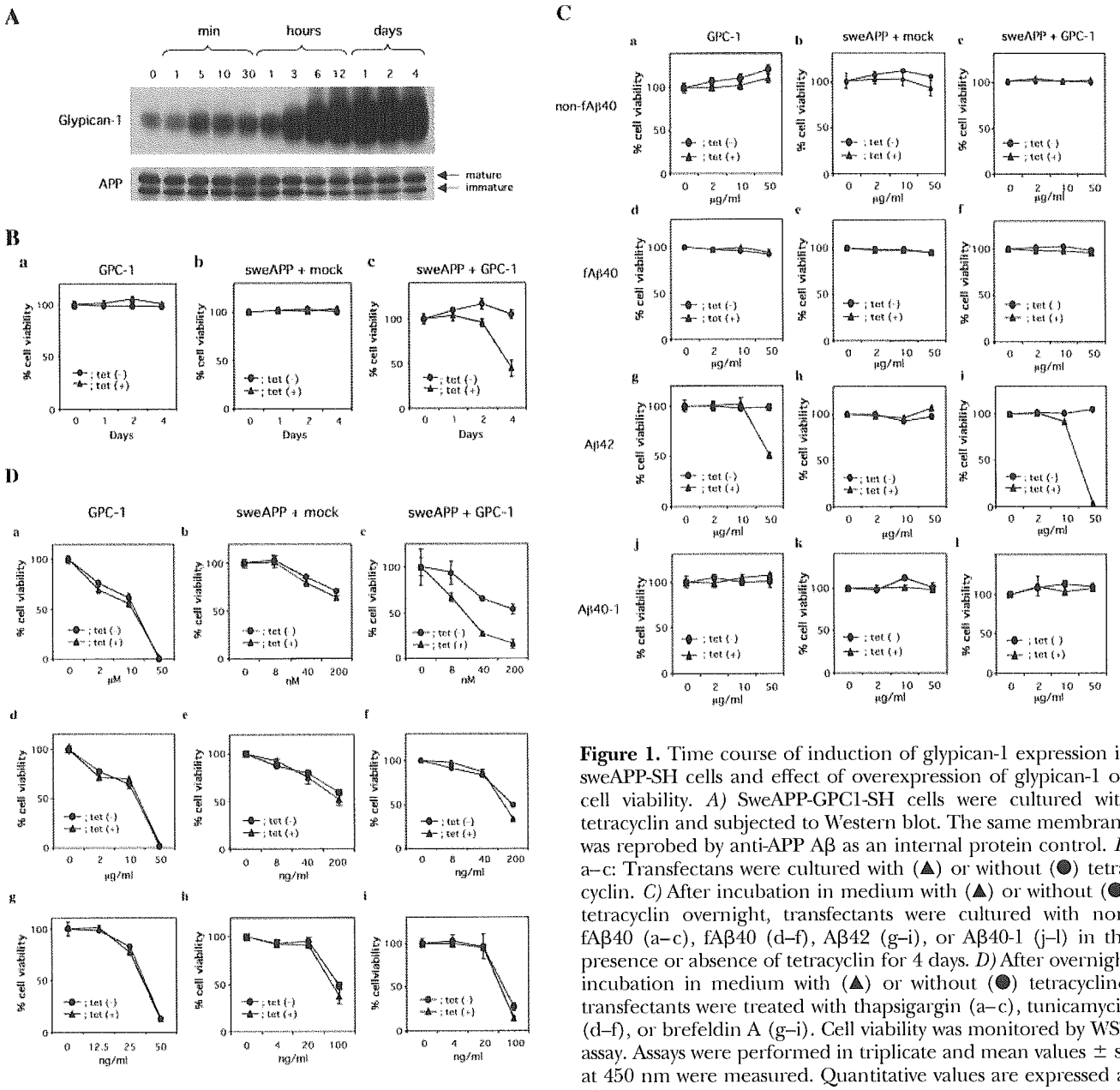

Figure 1. Time course of induction of glypican-1 expression in sweAPP-SH cells and effect of overexpression of glypican-1 on cell viability. *A)* SweAPP-GPC1-SH cells were cultured with tetracyclin and subjected to Western blot. The same membrane was reprobed by anti-APP Aβ as an internal protein control. *B)* a–c: Transfectans were cultured with (▲) or without (●) tetracyclin. *C)* After incubation in medium with (▲) or without (●) tetracyclin overnight, transfectants were cultured with non-fAβ40 (a–c), fAβ40 (d–f), Aβ42 (g–i), or Aβ40-1 (j–l) in the presence or absence of tetracyclin for 4 days. *D)* After overnight incubation in medium with (▲) or without (●) tetracycline, transfectants were treated with thapsigargin (a–c), tunicamycin (d–f), or brefeldin A (g–i). Cell viability was monitored by WST assay. Assays were performed in triplicate and mean values ± SE at 450 nm were measured. Quantitative values are expressed as % of cell viability in untreated cells.

brane domains called detergent-insoluble, glycosphingolipid-enriched (DIG) domains. We next examined whether glypican-1 is present in the DIG fraction from brain tissues. DIG was recovered in fractions 4–6, into which flotillin (a marker protein of DIG) was exclusively fractionated. Western blot with anti-glypican-1 showed that glypican-1 was mainly present in fraction 5, indicating localization of glypican-1 to the DIG domains. Western blot with 3G10 mAb indicated that HSPGs other than glypican-1 were almost undetectable in the DIG fraction. An insoluble pellet from the DIG fraction was solubilized with 6 M guanidine-HCl and the solubilized sample was used for dot blot Aβ binding assay. Aβ bound to guanidine soluble samples from the DIG fraction; this binding was significantly inhibited by heparitinase pretreatment, indicating that Aβ could bind to DIG-resided glypican-1 in an HS chain-dependent manner.

4. Co-localization of glypican-1 and Aβ in DIG fractions from AD brain

Recently, DIG domains or "rafts" have received attention with regard to the pathogenesis of AD, because accumulation of Aβ in these domains was demonstrated and appeared to correlate with the extent of Aβ deposition in the brain. Thus, it is possible that glypican-1 participates in the process of Aβ accumulation by interaction with Aβ in such specific microdomains. To clarify this, we examined whether glypican-1 and Aβ are co-fractionated in DIG fractions from AD brains. The fractions were analyzed by Western blots with antibodies to human glypican-1, Aβ, Aβ40, Aβ42, or APP. Glypican-1 was recovered mainly in DIG fractions. Full-length APP was fractionated predominantly in the high density fractions, and to a smaller extent in DIG fractions. We observed that signifi-

cant amounts of Aβ40 and Aβ42 were present in the DIG fractions as monomers and SDS stable dimers. BAN50, whose epitope is located in Aβ1-10, labeled Aβ monomers more strongly than Aβ dimers, suggesting these SDS stable dimers were formed in a way that the amino-terminal portion of Aβ was masked, modified, or deleted.

5. Preferential role of glypican-1 in Aβ42 accumulation in DIG domains

Given that Aβ binds to glypican-1 and these two proteins were accumulated in DIG domains, there may be a correlation between them. To examine this, we quantified levels of Aβ and glypican-1 in the DIG fraction using ELISA. There was a strong correlation between Aβ42 and glypican-1 (ctrl; r=0.9517, AD; r=0.8756) whereas no correlation between Aβ40 and glypican-1 (ctrl; r=0.3559, AD; r=0.0854) was observed. These results suggest that glypican-1 plays a preferential role in the accumulation of Aβ42 in DIG domains.

6. Effect of glypican-1 overexpression on cell viability

To explore the function of glypican-1 other than plaque formation, we generated transfectants that overexpressed glypican-1 in a tetracycline-inducible manner. Western blot analysis showed that the trasnsfectants were induced to express a large amount of glypican-1 protein when cultured with tetracyclin (**Fig. 1*A***). Time course experiments demonstrated that induction of glypican-1 expression was begun 5 min after addition of tetracyclin and reached maximal levels after 12 h. Cell viability of these cells with or without the induction of glypican-1 was analyzed using WST assay. As shown in Fig. 1, overexpression of glypican-1 decreased viability of cells coexpressing APP-carrying Swedish mutation of familial Alzheimer's disease (Fig. 1*Bc*). Neither glypican-1 nor Swedish APP overexpression alone affected cell viability (Fig. 1*Ba*, b). Since the production of Aβ in Swedish APP-expressing cells was increased, the observed effect of glypican-1 on cell viability may be due to enhanced Aβ toxicity by binding to glypican-1. To examine this, transfectants were cultured with exogenously added Aβ for 4 days, then cell viability was measured. The viability of all cells examined was not affected by adding non-fAβ40, fAβ40, or Aβ40-1 even though glypican-1 expression was induced (Fig. 1*Ca*–f, j–l). In contrast, Aβ42 significantly reduced cell viability only when glypican-1 was overexpressed (Fig. 1*Cg*, i). These results indicate that cells that overexpress glypican-1 become more susceptible to Aβ42 toxicity, resulting in enhanced cell death.

7. Effect of glypican-1 overexpression on ER stress

It has been reported that ER stress is an important factor in the neuropathology of a wide variety of neurological disorders, including AD. Studies have shown that neurotoxicity elicited by Aβ is at least partially mediated by ER,

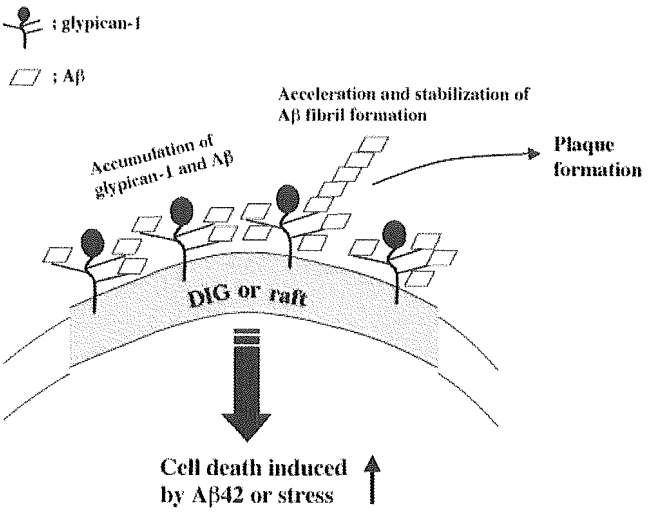

Figure 2. Proposed roles of glypican-1 in AD. Glypican-1 is involved in Aβ accumulation in DIG or raft domains; hence *1)* fibril and plaque formations are accelerated by binding to HS chains on glypican-1, and *2)* neuronal cell death induced by Aβ42 or certain stresses is enhanced when glypican-1 levels are increased.

raising the possibility that the ER stress response is influenced by overexpression of glypican-1, which may result in enhanced susceptibility of cells to Aβ42 toxicity. We examined the effect of glypican-1 expression on the stress response. Cell death induced by thapsigargin was accelerated when glypican-1 was overexpressed in cells coexpressing Swedish APP (Fig. 1*Dc*). Such an acceleration was not observed in cells that expressed glypican-1 or Swedish APP alone (Fig. 1*Da*, b). The stress response by tunicamycin and brefeldin A did not alter the cell survival even though cells were coexpressing Swedish APP and glypican-1 (Fig. 1*Dd*–i). These results suggest that glypican-1, together with Aβ, makes cells more vulnerable to some but not all stresses.

CONCLUSIONS AND SIGNIFICANCE

Although HSPGs are co-localized in senile plaques and may promote amyloid formation, deposition, and/or persistence by binding to Aβ, it remains uncertain how HSPGs are involved in AD pathogenesis and which HSPG has a pathogenic role in AD. The findings here suggest that glypican-1 binds to Aβ through HS chains and may be involved in accumulation of Aβ in DIG domains and/or the formation of plaques at an initial stage. Glypican-1 may act as a negative factor to neuronal cell survival, probably by binding with Aβ. Individuals whose expression levels of glypican-1 are relatively high might have a higher risk of AD. It is necessary to define more precisely the exact role of glypican-1 in these pathological events. A better understanding of normal and pathological functions of glypican-1 may lead to the development of new therapeutic approaches for AD. FJ

J Pharmacol Sci 97, 1 – 5 (2005)

Journal of Pharmacological Sciences

Current Perspective

Amyloid Pathology and Protein Kinase C (PKC): Possible Therapeutics Effects of PKC Activators

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Received October 18, 2004

Abstract. Amyloid β-protein (Aβ) is one of the most studied peptides in human neurodegenerative disorders. Although much has been learned about the biochemistry of this peptide, fundamental questions such as when and how the Aβ becomes pathologic remain unanswered. In this article we review the recent findings on the biology and pathology of Aβ and the role protein kinase C (PKC) plays in these processes. The potential neuroprotective role of PKC and the possible therapeutic effects of PKC activators in Alzheimer's disease (AD) will be discussed. Briefly, comments will be also addressed on the role of PKC in cell death and neurogenesis in AD.

Keywords: amyloid protein, Alzheimer's disease, signal transduction, protein kinase C, cell death

Amyloid β-protein localization: normal and pathologic

Amyloid β-protein (Aβ) has been identified initially as a 'pathologic' protein associated with the Alzheimer's dementia. Over the past two decades, extensive studies on the pathogenesis of Aβ have revealed that generation of soluble Aβ is a normal event. Evidence to date demonstrates that the protein is secreted by cells in normal conditions mainly as the 40 amino-acid fragment of Aβ (for review, see ref. 1). Aβ is present in the cerebrospinal fluid and plasma of normal subjects (for review, see ref. 1), but low and detectable amounts of Aβ40 can also be found in cortical tissue of young non-demented individuals (2). In contrast, in normal aged individuals (3) and in a group of elderly non-demented subjects, higher levels of insoluble Aβ42 are reported (2). These findings suggest that although Aβ is secreted throughout life, it begins to accumulate in old age. At the later stage of life, age-dependent biochemical alterations in concert with Alzheimer's disease (AD) risk factors could accelerate the deposition of readily aggregated Aβ42. In light of these observations, the amyloid hypothesis is currently being reconsidered and again supports a central role of Aβ in the pathophysiology of the AD.

It is now understood that neurons are the main source of Aβ in AD, although it has been reported that astroglia and microglia might synthesize or transport the soluble forms of Aβ from the interstitial space into the senile plaques or vascular walls (see ref. 2). However, the origin of the amyloid deposits identifiable in the extracellular parenchyma in AD is still debated. It has been hypothesized that Aβ gradually accumulates in the extracellular space due to excess secretion and/or deficient clearance (see ref. 2). It has also been suspected that Aβ initially aggregates and accumulates in the neuronal cell body and the cell deposits only become extracellular after the aggregates disrupt the cell integrity. In favor of the latter hypothesis, recent data have revealed the presence of aggregated Aβ42 within neuronal cell bodies by using specific antibodies against

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Invited article

the C-terminus of the Aβ42 molecule (4). The authors have also detected Aβ-like plaque staining that has neuronal shape, suggesting a neuronal origin of amyloid plaques (4). In addition, a new report convincingly describes intracellular Aβ42 immunolabeling in pyramidal neurons and discusses the technical artifacts in the current methods used to detect the amyloid plaques (5). Together these findings support the hypothesis that the extracellular amyloid plaques originate from the intraneuronal pool of accumulated Aβ42. If this is the case, one might suggest that the neuronal aggregation of Aβ42 is an initial and necessary event in the generation of plaques in AD. What triggers the aggregation and deposition of Aβ still remains unclear. Higher prevalence of sporadic rather than genetically-linked cases of AD suggests a random biochemical mechanism that leads to this process. Clinical data together with immunohistochemical analysis of plaques suggested a role for protein kinase C (PKC) in the pathogenesis of Aβ. Namely, previous studies have shown that both diffuse and mature plaques are immunoreactive for the isoform βII of PKC but not for PKC(α), PKC(βI), or PKC(γ), suggesting an early role of PKC(βII) in Aβ aggregation (for review, see ref. 6). Additional studies have shown that anti-PKC(βII) immunostaining is elevated in the diffuse plaques and more pronounced in mature plaques, while very low anti-PKC(βII) reactivity is found outside the deposits. Although, the high intensity of anti-PKC(βII) immunolabeling does not necessarily indicate high activity, the results are compatible with the hypothesis that neuronal hyperactivation at early stages may be involved in AD neuropathology (for review, see ref. 6).

Role of PKC in Aβ generation

PKC is a phospholipid-dependent protein kinase that plays a crucial role in various cellular functions in neuronal and non-neuronal cells. Molecular cloning has identified eleven PKC isoforms with individual characteristics and enriched distribution in the brain (7). In neurons, PKC is a key enzyme in neurotransmission, synaptic plasticity, and learning and memory. Recent in vitro data suggest that certain PKC isoforms are also intimately involved in cell survival (8). With regard to AD, PKC is linked to amyloid precursor protein (APP) processing, regulates the expression of APP mRNA, and as suggested by recent studies, may be involved in presenilin (PS) protein function as well (9).

Aβ is generated from APP, an integral membrane protein ubiquitously expressed in cells. APP is particularly abundant in neurons, but may also be present in astrocytes, microglia, and endothelial smooth muscle. APP can be cleaved at three internal sites by three distinct proteolytic enzymes: α-, β-, and γ-secretases (for review, see ref. 1). Cleavage by β- and γ-secretases at the N and C terminus of APP generates the amyloidogenic fragments of Aβ. The α-pathway is a non-amyloidogenic cascade that is controlled by PKC. α-Secretase enzyme cleaves APP at a site within the Aβ sequence and generates an extracellular soluble fragment (sAPPα) and an intracellular fragment C83, thus precluding the formation of an intact Aβ. These cleavages occur both in brain and in peripheral tissues in AD fibroblasts (10) and could take place in different subcellular organelles (for review, see ref. 11). In vitro studies have demonstrated that different PKC isoforms play distinct roles in APP processing. PKCα is specifically involved in phorbol ester-induced sAPPα release, while PKCε is involved in coupling cholinergic receptors with APP metabolism (12). In vivo studies using a guinea-pig model with constitutive overactivation of PKCα and -β isoforms have shown an increase in sAPPα production, indicating that these isoforms are key regulators of α-secretory APP processing (13). Despite of sAPPα increase in this animal model, additional studies have found no changes in tissue concentration of Aβ peptides in the neocortex or hippocampus, suggesting that generation of Aβ through the β-secretase pathway is independently regulated from the α-secretase pathway (14). These findings further suggest that it is questionable to use drug therapies based on PKC activators to reduce Aβ level because increased secretory APP processing does not necessarily affect Aβ generation or slow amyloid plaque formation. In contrast, other lines of evidence obtained in the mouse model have suggested that PKC activators might enhance secretion of APPα and reduce Aβ40 in the brains of APP[V717I] transgenic mice that overexpress the APP "London" mutant and develop abundant amyloid plaques later in life (15). The discrepancy between these studies could be explained by a cross-species variability of APP and Aβ. Namely, it has been found that in mice and rats, APP is less processed by the β-secretase pathway than in primates. The Aβ peptides in rodents differ in three amino acids from Aβ peptides in humans and display lower tendency to aggregate. In contrast, the APP molecule in guinea pig shares high degree of homology with human APP, its processing is very similar to that of human APP, and the Aβ sequence is identical to that of human Aβ (14). Additional in vivo evidence is necessary to clarify the relationship between secretory APP processing induced by PKC activation and Aβ generation.

The presenilin (PS) 1 and PS2 proteins are transmembrane proteins that are critically involved in γ-

secretase cleavage of APP (16). Mutations in the PS genes that cause familial forms of early onset Alzheimer's disease lead to increased production of Aβ42. Very recent data has identified a phosphorylation site for PKC within a recognition motif for caspases in PS1 (9). Phosphorylation at this site by PKC regulates the caspase-mediated cleavage of PS1 and inhibits the progression of apoptosis. These new findings support an anti-apoptotic role of PKC. Furthermore, different PKC isoforms seem to be involved in cell survival. Studies show that PKC(α) is able to phosphorylate Bcl-2, an anti-apoptotic protein, at a site that increases its anti-apoptotic function and overexpression of PKC(ε) results in increased expression of Bcl-2 (8).

Aβ accumulation and PKC signaling

Intraneuronal accumulation of Aβ occurs in normal aging without deposition of Aβ in amyloid plaques (3) and/or associated with extracellular deposits in the AD. The mechanisms underlying this process might be primarily related to a deficiency of an intracellular signal transduction system that develops with normal aging and is amplified by the AD risk factors. Immunohistochemical studies in aged non-demented subjects have shown that neurons with marked intracellular Aβ42 immunoreactivity also stain positive for apolipoprotein E (ApoE) (4), a major risk factor for late-onset of AD (17). A key signal transduction system that generally declines with aging is PKC. Consistent alterations of PKC level (18), PKC activity (19), and anchoring mechanisms for PKCs to subcellular compartments through receptors for activated C kinase (RACK1) (20) have also been reported in the postmortem tissue from AD brains. Not all aged individuals develop AD, but aging seems to be a prerequisite of AD. It can be hypothesized that in aging in the presence of high risk AD factors, PKC deficiency would unbalance the APP α-processing towards a β- and/or γ-processing with generation of soluble Aβ. Therefore, it is reasonable to think that gradual elevation of soluble Aβ will initially activate PKC and related down-stream pathways, while high constant level of Aβ, as in the late stage of AD, will downregulate PKC and dampens the PKC-related intracellular pathways. Clinical and experimental studies show hyperactivation of PKC in AD patients and in animal models. Increased immunoreactivity for PKC(βII) has been found in diffuse and senile amyloid plaques (6). A recent study in postmortem AD brain tissue has found increased mitogen-activated kinases (MAPK) activity, an intracellular enzyme located downstream to PKC that could be responsible for the enhanced phosphorylation of tau protein and subsequent generation of neurofibrillary tangles (NFT) (21). Transgenic mice that overexpress the Swedish mutant of human APP695, Tg2576, develop numerous cortical and hippocampal Aβ plaques at 9 – 13 months of age and show an activation of multiple isoforms of PKC involved in APP processing (PKCα) and neuronal survival (PKCγ, PKCζ) (22). In contrast, by 20 months of age, Tg2576 mice show decreased extracellular signal-regulated kinase (ERK2) MAPK and reduced activation of transcription factor cAMP-response element binding protein (CREB) (23). We have recently shown that chronic exogenous administration of soluble Aβ into the rat cerebral ventricle increases radioreceptor binding to phorbol dibutyrate (PDBu), a measure of enhanced PKC activation, and also decreases the activity and translocation of PKC to the cellular membrane in the hippocampus (24). The dual effect of Aβ on PKC in our experiment could be explained by a chronic delivering system (for review, see ref. 25). In the first days of infusion, low accumulation of Aβ could activate PKC enzyme but a continuous delivery system would increase the Aβ level that could stimulate permanently PKC and induce its down-regulation. The enzyme would be long-lasting bound to membrane and thus unable to respond to further stimulation. Decreased activity of PKC in our non-transgenic animal model is not secondary to an Aβ-induced neurodegeneration because studies on this model do not find cell death in the hippocampus (for review, see ref. 26). Rather we suggest that PKC alteration is a primary event in AD progression because other studies on peripheral tissue from AD patients have shown reduced PKC activity in non-neuronal cells such as fibroblasts (10). Ultimately, the decline in the intracellular signal transduction systems in AD that lead to decreased activation of transcription factors and protein synthesis may cause cell death and memory loss.

Cell death, neurogenesis, and Alzheimer's disease

Memory loss associated with late stages of AD is believed to be caused by neuronal degeneration in cognition-related brain regions. In the AD brain, large-scale cell death of mature neurons is a pathologic process that remains unsolved. Recently, new types of cell death have been proposed for neuronal loss specifically through apoptosis and cell cycle reentry. Aβ protein could be a driving force in these processes. The amyloid protein induces apoptosis through oxidative stress while also driving cell division and cell death in cultured neurons (27). Evidence for DNA fragmentation, expression of apoptosis-related genes, and caspase activation support an apoptotic mechanism in AD neurodegenera-

tion. Studies have shown that several atypical isoforms of PKC might suppress apoptosis induced by Aβ and promote survival (8). Potential therapeutic agents for AD based on PKC activators have been also proposed in clinical studies (15). Very recent lines of evidence have suggested that mature neurons degenerate in AD and in mild cognitive impairment (MCI) because they reenter a cell cycle they are unable to complete (28). Immunohistochemical data from AD tissue has revealed reexpression of cell cycle related-proteins in neurons undergoing cell death. There is also direct evidence that neurons in AD enter the cell cycle but do not complete mitosis and remain tetraploid. These poplyploid neurons survive for several months after their genome is replicated but ultimately die through an apoptotic process. To date there is no direct link between cell cycle activation and PKC, but cell culture studies have shown that PKC along with other kinases is required for vascular endothelial growth factor (VEGF)-induced proliferation of neuronal precursors (29).

Although AD is characterized by neuronal degeneration and cell loss, recent studies have revealed increased neurogenesis in the dentate gyrus and CA1 area in AD brains (30). Increased generation of new neurons in the hippocampus could be a secondary event to the massive cell loss observed with the progression of the disease. Normal aged brain has markedly reduced hippocampal neurogenesis but interestingly, preserves the potential to increase the production of new neurons in certain conditions (31). Alternatively, neurogenesis might be directly stimulated by Aβ in AD brains. Determining which mechanism underlies the enhanced neurogenesis in AD brain needs further elucidation, but these findings leave open the question whether the stem cell-based therapies in AD could be a practical therapeutic issue to focus on.

Conclusions

Aβ has a central role in the pathogenesis of AD. Generation of non-amyloidogenic fragments of the amyloid protein is controlled by PKC, an enzyme that is downregulated during aging. In elderly individuals, decreased PKC activity in concert with increased AD risk factors could set up the stage for accumulation of Aβ in neurons and progression of AD. Consequently, decreased intracellular signaling pathways controlled by PKC could reduce neuronal survival, increase cell death, and induce memory loss in AD. Drugs developed as PKC activators are of potential therapeutic interest for AD but additional studies are needed to support this idea. Recent findings that the AD brain is able to generate more new neurons than the normal adult brain bring also a new and exciting view into stem cell therapy. The mechanisms that control this process must still be identified and more importantly, whether it can be translated into a practical approach for the AD therapy.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Science Research (No. 14370031) and a COE grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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α7-Type Nicotinic Acetylcholine Receptor and Prodynorphin mRNA Expression after Administration of (–)-Nicotine and U-50,488H in β-Amyloid Peptide (25-35)–Treated Mice

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ABSTRACT: **We previously reported that (–)-nicotine and κ-opioid receptor agonists lessened impairment of learning and/or memory in several animal models. Furthermore, these drugs prevented neurodegenerative damage induced by ischemia or β-amyloid peptide (25-35). In the present study, we tested whether (–)-nicotine and U-50,488H prevent delayed-memory impairment induced by β-amyloid peptide (25-35), and changes of expression of α7-type nicotinic acetylcholine receptor mRNA and prodynorphin mRNA. Seven days after treatment with β-amyloid peptide (25-35) (9 nmol/mouse, i.c.v.), memory impairment was observed in the Y-maze test. Memory impairment was prevented when (–)-nicotine (6.16 μmol/kg, s.c.) or U-50,488H (21 μmol/kg, s.c.) was administered 1 h before, but not 1 h after, β-amyloid peptide (25-35) treatment. There was no change in prodynorphin mRNA or α7-type nicotinic acetylcholine receptor mRNA expression in the hippocampus 10 days after β-amyloid peptide (25-35) treatment alone. Of interest, mRNA expression of not only prodynorphin, but also the α7-type nicotinic acetylcholine receptor, was significantly decreased when U-50,488H was administered 1 h before, but not 1 h after, treatment with β-amyloid peptide (25-35). However, these changes were not observed after the administration of (–)-nicotine. These results suggest that activation of the κ-opioid system, but not α7-type nicotinic receptors has a neuroprotective effect on β-amyloid peptide (25-35)–induced memory impairment, and may be involved in the long-lasting changes in the expression of these mRNAs.**

KEYWORDS: **(–)-nicotine; U-50,488H; kappa-opioid receptor; beta-amyloid peptide (25-35); alpha7 nicotinic acetylcholine receptor mRNA; prodynorphin mRNA; learning and memory; neurodegeneration**

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Ann. N.Y. Acad. Sci. 1025: 508–514 (2004).
doi: 10.1196/annals.1316.063

INTRODUCTION

Cholinergic neuronal systems play an important role in the cognitive deficits associated with aging and neurodegenerative diseases. In the brains of patients with Alzheimer's disease, large decreases in the number of not only muscarinic binding sites, but also nicotinic sites have been reported.[1–3] (–)-Nicotine improves the performance of cognitively demanding tasks in normal subjects[4] and information processing and attention in patients with Alzheimer's disease.[5] Moreover, not only acute (–)-nicotine treatment[6–8] in rats and mice, but also chronic (–)-nicotine pretreatment reduced cholinergic dysfunction, and learning and memory impairment in animal models such as basal forebrain–lesioned rats and β-amyloid protein–infused rats.[9,10] Hippocampal synaptic transmission is enhanced by low concentrations of nicotine, which seems to contribute to the enhancement of memory.[8,11]

The opioid peptide dynorphin A has been examined in numerous models in order to determine its functional role in the CNS. The effects produced by exogenously applied dynorphins vary from neurotoxicity[12] and impairment of retention of inhibitory avoidance[13] to neuroprotection,[14,15] and improvement of learning and/or memory.[15–18] The selective κ-opioid receptor agonist U-50,488H prevented ischemia-induced memory impairment.[15] We previously reported that dynorphin A-(1-13) and U-50,488H improved carbon monoxide– and β-amyloid peptide (25-35)–induced delayed impairment of learning and/or memory in mice.[16,19] As described above, while κ-opioid agonists such as U-50,488H and dynorphin A-(1-13) are known to have neuroprotective effects, the mechanism by which these drugs improve β-amyloid peptide (25-35)–induced delayed impairment of learning and memory is still unclear. These findings may indicate that the neuroprotective effects of (–)-nicotine and κ-opioid agonists involve common mechanisms of action.

An animal model in which a long-term cholinergic hypofunction has been pharmacologically induced would be useful for evaluating new cholinergic therapies. Several observations following β-amyloid peptide (25-35) injection are of particular relevance for understanding the pathogenesis and progression of Alzheimer's disease.[6,19] These observations indicate that the β-amyloid peptide (25-35)–induced cholinergic deficit in the brain represents a reliable tool with which to study several mechanisms possibly involved in Alzheimer's disease.

In this study, we tested whether (–)-nicotine and U-50,488H prevent delayed-memory impairment induced by β-amyloid peptide (25-35), and changes of expression of α7-type nicotinic acetylcholine receptor mRNA and prodynorphin mRNA.

MATERIALS AND METHODS

Animals

Seven-week-old male ddY mice (Japan SLC, Japan) were kept in a controlled environment, with controlled lighting (12-h light/dark cycle, lights on; 8 AM to 8 PM) and temperature (23 ± 2°C) for at least 5 days before the experiments, and were given free access to food and water.

Drugs

The following drugs were used: β-amyloid peptide (25-35) (Sigma, St. Louis, MO), *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl] cyclohexyl) benzeneacetamide methanesulfonate (U-50,488H, Sigma); and (–)-nicotine hydrogen tartrate salt [(–)-nicotine; Sigma]. All doses were calculated as those of the bases. β-amyloid peptide (25-35) was dissolved in sterile double-distilled water at a concentration of 1 mg/mL and stored at –20°C. β-Amyloid peptide (25-35) was aggregated or "aged" by incubation in sterile distilled water at 37°C for 4 days, as described by Maurice *et al.*[6] All other drugs were dissolved in isotonic saline solution (Otsuka Pharmaceuticals, Inc., Tokyo, Japan). U-50,488H and (–)-nicotine were administered 1 h before and/or 1 h after β-amyloid peptide (25-35) injection. β-Amyloid peptide (25-35) was injected into the lateral ventricullar (i.c.v.) region of the mouse brain according to the method of Haley and McCormick[20] in a volume of 5 μL/mouse with the animal under brief ether anesthesia.

Spontaneous Alternation Performance

Immediate working memory performance was assessed by recording spontaneous alternation behavior in a single session in a Y-maze. Each mouse, new to the maze, was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The series of arm entries was recorded visually. Entry was considered to be completed when the hind paws of the mouse had been completely placed in the arm. Alternation was defined as successive entries into the three different arms (A, B, or C) on overlapping triplet sets (e.g., ACBABACBAB = 5). Percentage alternation was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries minus two), multiplied by 100 as shown in the following equation:

$$\% \text{ Alternation} = [(\text{Number of alternations})/(\text{Total arm entries} - 2)] \times 100$$

Alternation performance was assessed 7 days after β-amyloid injection.

Northern Blot Analyses

Mice were sacrificed and brains were removed. The hippocampus was dissected out and used for preparation of total RNA for Northern blot analysis. Total (10 μg) RNA was fractionated on a 1% agarose, 0.7 % formaldehyde denaturing gel, and the RNA fractions were transferred overnight to a nylon membrane. The membrane was hybridized with [(alpha)-^{32}P]dCTP-labeled cDNA of prodynorphin or α7 nicotinic acetylcholine receptor overnight at 42 °C in 40 % formamide, 10% dextran sulfate, 4 × SSC (1 × SSC contained 150 mM NaCl and 15 mM sodium citrate [pH 7.0]), 7 mM Tris/HCl (pH 7.6), 0.8 × Denhardt's solution, 20 μg/mL salmon sperm DNA, and 0.5% sodium dodecyl sulfate (SDS). After hybridization, the membrane was washed twice at room temperature (2 × SSC and 0.1% SDS for 20 min) and once at 60°C (0.2 × SSC and 0.1% SDS for 20 min) and then autoradiographed. Hybridization of the membrane was performed by reprobing the same Northern blot membrane with each full-length cDNA probe after washing at high stringency (80°C, 10 mM Tris/HCl [pH 7.0], and 0.01% SDS).

GAPDH or β-actin was used to normalize the expression of prodynorphin and α7 nicotinic acetylcholine receptor mRNA. Optical density in autoradiogphed membranes was calculated using a GelDoc 2000 image analyzing system (BioRad).

Data Analysis

The behavioral data were expressed in terms of the median and interquartile ranges. The significance of differences was evaluated using the Mann-Whitney U test for comparisons between two groups and the Kruskal-Wallis non-parametric one-way analysis of variance followed by Bonferroni's test for multiple comparisons. The mRNA levels were expressed in terms of means and SE. The significance of differences was evaluated using a one-way analysis of variance followed by Bonferroni's test for multiple comparisons. The criterion for significance was $P < .05$ in all statistical evaluations.

RESULTS

Effects of U-50,488H and (–)-Nicotine on β-Amyloid Peptide (25-35)–Induced Impairment of Spontaneous Alternation Behavior in Mice

β-Amyloid peptide (25-35) (9 nmol/mouse, i.c.v.) was found to induce delayed impairment of spontaneous alternation behavior when the Y-maze test was carried out 7 days after i.c.v. injection. Delayed impairment of spontaneous alternation was prevented when U-50,488H (21 μmol/kg, s.c.) was administered 1 h before, but not 1 h after, the β-amyloid peptide (25-35) (TABLE 1).

Delayed impairment of spontaneous alternation was prevented significantly when (–)-nicotine (6.16 μmol/kg, s.c.) was administered 1 h before, but not 1 h after, the β-amyloid peptide (25-35) (TABLE 1).

Effects of U-50,488H on α7 Nicotinic Acetylcholine Receptor and Prodynorphin mRNA Expression in β-Amyloid Peptide (25-35)–Treated Mouse Hippocampus

When U-50,488H (21 μmol/kg, s.c.) was administered 1 h before the β-amyloid peptide (25-35), α7 nicotinic acetylcholine receptor mRNA expression was signifi-

TABLE 1. Summary of results

Treatments	Administration 1 hour before	Administration 1 hour after	Alternation (%)	Prodynorphan mRNA	α7 Nicotinic ACh Receptor mRNA
β-amyloid peptide (25-35)			Impaired	±	±
U-50,488H	+	–	Prevented	Decreased	Decreased
	–	+	±	±	±
(–)-nicotine	+	–	Prevented	±	±
	–	+	±	±	±

cantly decreased after 10 days. However, there was no significant effect when U-50,488H was administered 1 h after injection of the β-amyloid peptide (25-35).

Prodynorphin mRNA expression was significantly decreased, when U-50,488H was administered 1 h before, but not 1 h after, the β-amyloid peptide (25-35) (TABLE 1).

Effects of (–)-Nicotine on α7 Nicotinic Acetylcholine Receptor and Prodynorphin mRNA Expression in β-Amyloid Peptide (25-35)–Treated Mouse Hippocampus

When (–)-nicotine (6.16 μmoL/kg, s.c.) was administered 1 h before or 1 h after the β-amyloid peptide (25-35), there was no significant change in α7 nicotinic acetylcholine receptor and prodynorphin mRNA expression 10 days later (TABLE 1).

DISCUSSION

In 26-month-old transgenic mice overexpressing the human β-amyloid precursor protein, dynorphin levels in mossy fibers of the hippocampus increased markedly.[21] As described in the introduction, a selective κ-opioid receptor agonist, U-50,488H, prevented ischemia-induced memory impairment.[15] Furthermore, we previously demonstrated that dynorphin A-(1-13) or U-50,488H improved carbon monoxide- and β-amyloid peptide (25-35)–induced delayed impairment of learning and/or memory in mice.[16,19] Taken together, it is speculated that κ-opioid receptor agonists have important roles in neuroprotection and the improvement of impaired learning and/or memory function. The present results indicated that pretreatment with U-50,488H significantly prevented β-amyloid peptide (25-35)–induced memory impairment, but U-50,488H no longer had a neuroprotective effect when given 1 h after the β-amyloid peptide (25-35) treatment. Prodynorphin mRNA expression in the hippocampus 10 days after β-amyloid peptide (25-35) treatment tended to increase. The mRNA expression of prodynorphin was significantly decreased when U-50,488H was administered 1 h before, but not 1 h after, β-amyloid peptide (25-35) treatment. These findings suggest that preactivation of the κ-opioid neuronal circuit is necessary for a neuroprotective effect.

We recently reported that repeated administration of (–)-nicotine prevented the impairment of performance induced by AF-64A treatment in the passive avoidance task.[22] A previous report showed that U-50,488H prevented ischemia-induced memory impairment.[15] Therefore, these findings led us to speculate that the neuroprotective effect of (–)-nicotine has a common mechanism of action with the effect of κ-opioid agonists.

Memory impairment was prevented when (–)-nicotine (6.16 μmol/kg, s.c.) was administered 1 h before, but not 1 h after, β-amyloid peptide (25-35) treatment, similar to the case for U-50,488H described above. mRNA expression of the α7-type nicotinic acetylcholine receptor was significantly decreased when U-50,488H was administered 1 h before, but not 1 h after, β-amyloid peptide (25-35) treatment. There was no change in α7 nicotinic acetylcholine receptor mRNA expression in the hippocampus 10 days after β-amyloid peptide (25-35) treatment. No significant change in prodynorphin and α7-type nicotinic acetylcholine receptor mRNA levels

was observed after (–)-nicotine administration in β-amyloid peptide (25-35)–treated mice. The decreased number of nicotinic receptor binding sites due to the toxicity of the β-amyloid peptide (25-35) was prevented by the addition of nicotine at low concentrations.[23] It was reported that a reasonably low concentration of nicotine acts as an antioxidant and plays an important role in the neuroprotective effect.[23] Taken together, (–)-nicotine has an important role in neuroprotection, but the underlying mechanism may not be the same as that for κ-opioid receptor agonists.

In conclusion, although the precise mechanism of neuroprotection is still unknown, activation of the κ-opioid system and/or nicotinic receptors may produce neuroprotective effects on β-amyloid peptide (25-35)–induced memory impairment. Since only the activation of the κ-opioid system produced long-lasting changes in the mRNA expression, the mechanisms of action of these drugs may be different.

ACKNOWLEDGMENTS

This study was supported in part by the Japan Smoking Research Foundation and Scientific Frontier Research Project of Meijo University from the Ministry of Education, Science, Sports and Culture, Japan.

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European Journal of Pharmacology 486 (2004) 151–161
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Enhanced antidepressant efficacy of σ_1 receptor agonists in rats after chronic intracerebroventricular infusion of β-amyloid-(1–40) protein

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Received 1 September 2003; received in revised form 4 December 2003; accepted 12 December 2003

Abstract

Treatment of depressive symptoms in patients suffering from neurodegenerative disorders remains a challenging issue, since few available antidepressants present an adequate efficacy during pathological aging. Previous reports suggested that selective σ_1 receptor agonists might constitute putative candidates. We here examined the pharmacological efficacy of igmesine and (+)-SKF-10,047 and the σ_1 receptor-related neuroactive steroid dehydroepiandrosterone sulfate, in rats infused intracerebroventricularly during 14 days with the β-amyloid-(1–40) protein and then submitted to the conditioned fear stress test. Igmesine and (+)-SKF-10,047 significantly reduced the stress-induced motor suppression at 30 and 6 mg/kg, respectively, in β-amyloid-(40–1)-treated control rats. Active doses were decreased, to 10 and 3 mg/kg, respectively, in β-amyloid-(1–40)-treated animals. The dehydroepiandrosterone sulfate effect was also facilitated, both in dose (10 vs. 30 mg/kg) and intensity, in β-amyloid-(1–40)-treated rats. Neurosteroid levels were measured in several brain structures after β-amyloid infusion, in basal and stress conditions. Progesterone levels, both under basal and stress-induced conditions, were decreased in the hippocampus and cortex of β-amyloid-(1–40)-treated rats. The levels in pregnenolone, dehydroepiandrosterone and their sulfate esters appeared less affected by the β-amyloid infusion. The σ_1 receptor agonist efficacy is known to be inversely correlated to brain progesterone levels, synthesized mainly by neurons that are mainly affected by the β-amyloid toxicity. The present study suggests that σ_1 receptor agonists, due to their enhanced efficacy in a nontransgenic animal model, may alleviate Alzheimer's disease-associated depressive symptoms.

Keywords: σ_1 Receptor; Neuro(active)steroid; β-Amyloid-(1–40) protein; Alzheimer's disease; Depression; Conditioned fear stress; (Rat)

1. Introduction

Alzheimer's disease is the most common form of dementia among the elderly (Evans et al., 1989). Physiopathological features characteristic of Alzheimer's disease include abnormal extracellular accumulation of β-amyloid proteins into sensitive structures of the brain. Gradual deposition of β-amyloid protein in the form of neurotic plaques, apparition of neurofibrillary tangles as well as progressive cognitive deficits accompany the emergence of Alzheimer's disease (Selkoe, 1991). Among the most common and important complications of Alzheimer's disease is clinically relevant depression, which worsens patient disability and suffering. Prevalence of depression among Alzheimer's disease patients ranges from 0% to 86% depending on reports, but

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doi:10.1016/j.ejphar.2003.12.018

is usually considered to average about 30–50% (Zubenko and Moossy, 1988; Wragg and Jeste, 1989; Zubenko, 2000; Purandare et al., 2001; Olin et al., 2002). Depression could also be observed in association with other types of degenerative dementia, such as Parkinson's disease, Huntington's chorea, Pick's disease or vascular dementia (Zubenko and Moossy, 1988). The cause of depression observed in Alzheimer's disease patients seems to be less genetic and more structural, related to functional declines, than classic depression seen in adults (Boland, 2000; Espiritu et al., 2001). In particular, the neurodegenerative pathologies provoke anatomic damages, reduction of cerebral blood flow or receptor dysfunctions in particular brain structures, including frontotemporal areas, hippocampus or the locus cœruleus (Zubenko and Moossy, 1988; Forstl et al., 1992; Sheline et al., 1996). Alzheimer's disease patients developing major depression thus show a particular neuropathological and neurochemical context, mainly characterized by a severe central cholinergic deficit, occurring in basal forebrain structures innervating the hippocampus and neocortex (Zubenko and Moossy, 1988; Forstl et al., 1992; Sheline et al., 1996). Present therapeutic strategies using classical antidepressant treatments have produced contradictory findings and did not satisfactorily lead to clear depression reduction (Boland, 2000; Lyketsos et al., 2003; Zubenko et al., 2003). Novel therapeutic approaches with preserved antidepressant efficacy are thus needed to treat depression in patients with neurodegenerative dementia.

We have demonstrated that chronic administration of β-amyloid protein into the cerebral ventricle, using a long-term mini-pump implantation, produced memory impairments without apparent neurodegeneration (Nitta et al., 1994; Yamada and Nabeshima, 2000; Tran et al., 2002). However, numerous neurochemical and neurophysiological alterations were observed after infusion of β-amyloid protein, such as impairment of long-term potentiation (Itoh et al., 1999); functional reduction of cholinergic and dopaminergic systems (Itoh et al., 1996); changes in the ciliary neurotrophic factors levels (Yamada et al., 1995); changes in the mRNA expression of brain-derived neurotrophic factor (BDNF) (Tang et al., 2000); induction of inducible nitric oxide (NO) synthase (iNOS) and overproduction of NO in the hippocampus (Tran et al., 2001); tyrosine nitration of synaptophysin (Tran et al., 2003); impairment of endogenous antioxidant system (Kim et al., 2003); and reduced activation of protein kinase C (PKC) (Olariu et al., 2002). These observations suggested in a convergent manner that β-amyloid toxicity resulted in functional deficits affecting neuronal responses and signalling pathways within the hippocampus, sustaining the marked memory impairments.

The σ_1 receptor agonists are potent antidepressant drugs acting through a unique mechanism that suggests their potential efficacy in Alzheimer's disease-related depression (Maurice, 2002). The σ_1 receptor is a 223-amino acid protein, cloned in several animal species (Hanner et al., 1996; Kekuda et al., 1996) that appeared devoid of analogy with any other known mammalian protein. Selective σ_1 receptor ligands exert a potent neuromodulation on intracellular Ca^{2+} mobilisations and excitatory neurotransmitter systems, including noradrenergic, glutamatergic and cholinergic responses (for review, see Maurice et al., 1999). Its endogenous effector remains unidentified, but the biological relevance of this receptor is supported by the correlation observed between the σ_1 binding affinity and functional and behavioural effects of drugs, and the interaction of several endogenous systems with this receptor, including peptides of the neuropeptide Y family, or neuro(active)steroids. In particular, pregnenolone or dehydroepiandrosterone behaved as σ_1 receptor agonists, while progesterone is a potent antagonist (Maurice et al., 1999). A similar crossed pharmacology between neuro(active)steroids and σ_1 receptor ligands has been observed in animal models of depression, forced swimming or conditioned fear stress, or in the σ_1 receptor involvement in cocaine-induced conditioned place preference (Noda et al., 2000; Urani et al., 2001; Romieu et al., 2003).

In a previous study, we have reported that the antidepressant-like efficacy of igmesine or PRE-084, two σ_1 receptor agonists, measured using the forced swim test, were potentiated in mice injected intracerebroventricularly (i.c.v.) with β_{25-35}-amyloid peptide (Urani et al., 2002). This enhanced efficacy was attributed to decreased progesterone levels in the hippocampus of β_{25-35} animals and suggested that σ_1 agonists, due to their enhanced efficacy, may allow to alleviate the depressive symptoms associated with Alzheimer's disease (Urani et al., 2002). In the present study, these observations were confirmed and extended using the Alzheimer's disease model of rats chronically infused with β-amyloid-(1–40) protein (Nitta et al., 1994, 1997; Yamada et al., 1995, 1999). We examined the antidepressant-like efficacy of σ_1 receptor agonists, and particularly igmesine and the σ_1 receptor-related neuroactive steroid dehydroepiandrosterone sulfate, on the conditioned fear stress response of rats, as previously reported (Nabeshima et al., 1985; Kamei et al., 1997). The effect of the β-amyloid infusion on brain neurosteroid levels was measured in basal and stressful conditions.

2. Materials and methods

2.1. Animals and treatment

Male Wistar rats (Charles River Japan, Yokohama, Japan or breeding centre of the Faculty of Pharmacy, Montpellier, France) weighing 200–230 g at the beginning of the experiments were used. They were housed two or three per cage under standard light–dark conditions (12-h light cycle starting at 08:00 h) at a constant temperature of 23 ± 1 °C. The animals had free access to food and water and they have been handled in accordance with the guidelines established