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IGF-1 promotes β-amyloid production by a secretase-independent mechanism

Wataru Araki [a,*], Hideaki Kume [a], Akiko Oda [a,b], Akira Tamaoka [a,b], Fuyuki Kametani [c]

[a] Department of Demyelinating Disease and Aging, National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan
[b] Department of Neurology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan
[c] Tokyo Institute of Psychiatry, Tokyo Metropolitan Organization for Medical Research, Setagaya, Tokyo 156-8585, Japan

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ABSTRACT

β-Amyloid peptide (Aβ) is generated via the sequential proteolysis of β-amyloid precursor protein (APP) by β- and γ-secretases, and plays a crucial role in the pathogenesis of Alzheimer's disease (AD). Here, we sought to clarify the role of insulin-like growth factor-1 (IGF-1), implicated in the AD pathomechanism, in the generation of Aβ. Treatment of neuroblastoma SH-SY5Y cells expressing AD-associated Swedish mutant APP with IGF-1 did not alter cellular levels of APP, but significantly increased those of β-C-terminal fragment (β-CTF) and secreted Aβ. IGF-1 also enhanced APP phosphorylation at Thr668. Treatment of β-CTF-expressing cells with IGF-1 increased the levels of β-CTF and secreted Aβ. The IGF-1-induced augmentation of β-CTF was observed in the presence of γ-secretase inhibitors, but not in cells expressing β-CTF with a Thr668 to alanine substitution. These results suggest that IGF-1 promotes Aβ production through a secretase-independent mechanism involving APP phosphorylation.

Cerebral accumulation of β-amyloid peptide (Aβ) is a major neuropathological hallmark of Alzheimer's disease (AD) [1]. Aβ is produced by the sequential cleavage of the transmembrane amyloid precursor protein (APP) by β-secretase and γ-secretase, which have been identified as β-site APP cleaving enzyme 1 (BACE1) and presenilin 1 (or presenilin 2) complex, respectively [2]. In the amyloidogenic processing pathway, β-secretase cleavage of APP generates secreted APP (sAPP)-β (sAPP-β) and β-C-terminal fragment (β-CTF), the latter of which is then cleaved intramembranously by γ-secretase to produce Aβ. By comparison, non-amyloidogenic α-secretase processing of APP within the Aβ sequence generates sAPP-α and α-CTF, precluding Aβ production [1].

IGF-1 is a well-characterized trophic factor that regulates cell growth and survival. IGF-1 and its receptors are expressed in the brain, and IGF-1 is actively transported across the blood-brain barrier [3,4]. IGF-1 is known to exert neuroprotective effects and to modulate neuronal activity [5]. Several recent studies have implicated the IGF-1/insulin system in the AD pathomechanism [6,7]. In one study, insulin was found to stimulate Aβ release from neurons [8]. In other works, abnormalities in insulin and IGF-1 gene expression were detected in AD brains [9], and serum IGF-1 levels were found to be reduced in AD patients [10,11]. However, it remains unclear whether IGF-1 plays a specific role in the modulation of Aβ production. Here, we sought to clarify this issue, using human neuroblastoma SH-SY5Y cells expressing Swedish mutant APP (swAPP) or β-CTF. Our data suggest that IGF-1 promotes Aβ production through a secretase-independent mechanism involving APP phosphorylation.

Materials and methods

cDNA constructs. APP β-CTF cDNA was subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), as described previously [12,13]. APP β-CTF cDNA with the Thr668Ala mutation (APP695 isoform numbering) was generated using the GeneEditor™ in vitro mutagenesis system (Promega. Madison, WI, USA) according to the manufacturer's instructions.

Cell culture and transfection. Human neuroblastoma SH-SY5Y cells were cultured as described previously [14]. SH-SY5Y cells stably expressing swAPP were established previously [14]. SH-SY5Y cells were transfected with APP β-CTF or the Thr668Ala mutant APP β-CTF cDNA by the calcium phosphate method, and stable transformants were selected with 400 μg/mL G418.

Antibodies and chemicals. A rabbit polyclonal antibody specific for the C-terminus of APP was described previously [15]. A rabbit polyclonal antibody (P-Thr668 antibody) specific for the Thr668-phosphorylated APP (P-APP) was obtained from Cell Signaling Technology (Beverly, MA, USA). A rabbit polyclonal antibody against BACE1 and a mouse monoclonal antibody against β-actin were purchased from Chemicon (Temecula, CA, USA) and Sigma (St. Louis, MO, USA), respectively. IGF-1 was obtained from Invitrogen, and *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester (DAPT) [16] and L-685,458 [17] were from Calbiochem (San Diego, CA, USA).

* Corresponding author. Fax: +81 423 46 1747.
E-mail address: araki@ncnp.go.jp (W. Araki).

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Western blot analysis. Western blot analyses were performed as described previously [14,18]. Cells were lysed in RIPA buffer with protease inhibitors [18]. Proteins were separated on polyacrylamide gels and blotted onto polyvinylidene difluoride (PVDF) membranes. Membranes were treated with PBS containing 0.05% Tween-20 and 5% non-fat dry milk to block non-specific binding sites and were then probed with the appropriate antibodies. Membranes were subsequently incubated with anti-rabbit or anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase. Immunoreactive proteins were detected with chemiluminescence reagents (PerkinElmer, Boston, MA, USA). The protein bands were analyzed by densitometry for quantitative measurement of protein expression intensity with an image analyzer LAS-1000 (Fuji Film Co., Tokyo, Japan).

Aβ measurement. Cells were seeded into 12-well plates at a density of 5×10^5 cells/well and incubated in fresh growth medium with or without IGF-1 for 24 h. The concentrations of Aβ40 and Aβ42 in the media were measured essentially as described previously using sandwich ELISA kits (Wako, Osaka, Japan) [18].

Results

To examine whether IGF-1 affects Aβ production, we first used neuroblastoma SH-SY5Y cells expressing swAPP (designated SH-swAPP cells), which secreted large amounts of Aβ [14]. SH-SY5Y cells have been shown to express IGF-1 receptors [19]. We found that the amounts of Aβ40 and Aβ42 secreted by SH-swAPP cells treated with 50 or 100 ng/mL IGF-I for 1 day were ~150% and 140% or ~170% and 140% of the amounts secreted by untreated control cells, respectively (Fig. 1A). This Aβ-promoting effect of IGF-1 appeared to be dose-dependent. By comparison, the amounts of Aβ40 and Aβ42 secreted by cells treated with 50 ng/mL brain-derived neurotrophic factor (BDNF) were comparable to those secreted by control cells (data not shown). Western blot analysis of cell lysates revealed that cellular levels of APP and BACE1 were not altered, but levels of β-CTF detected by the APP C-terminal antibody were significantly augmented in cells treated with IGF-1, compared with untreated cells (Fig. 1B and C). Levels of α-CTF were similarly elevated in IGF-1-treated cells. In addition, Western blot analysis using the P-Thr668 antibody showed that Thr668-phosphorylated APP (P-APP) significantly increased by ~40–50% in IGF-1-treated cells, compared with the control (Fig. 1B and D). Such increases in the APP CTFs and APP phosphorylation at Thr668 were not observed in cells treated with BDNF (data not shown). We also examined sAPP secretion by immunoprecipitation-Western blot analysis [20], but no significant differences were observed in the amounts of total sAPP and sAPP-α between control and IGF-1-treated cells (data not shown).

To investigate the mechanisms by which IGF-1 increases APP CTF levels and Aβ production, we used SH-SY5Y cells expressing β-CTF (designated SH-β-CTF cells). Western blot analysis revealed that levels of β-CTF were significantly augmented in IGF-1-treated cells, and that this effect was dose-dependent (Fig. 2A and B). IGF-1

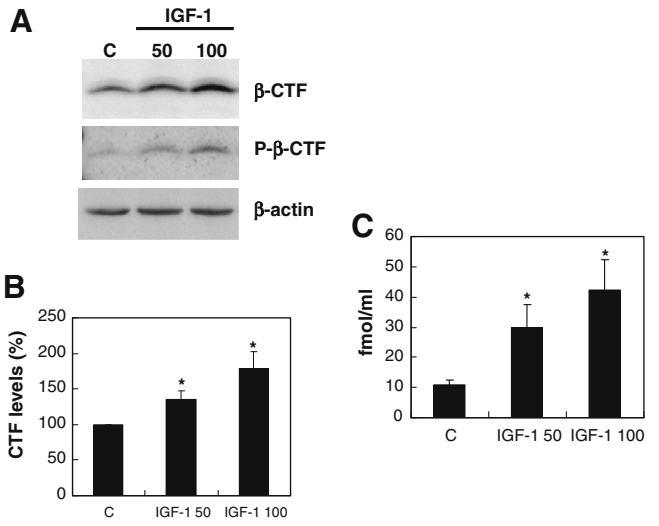

Fig. 2. Effects of IGF-1 on β-CTF levels and Aβ production in β-CTF-expressing cells. (A) SH-β-CTF cells were treated with or without IGF-1 for 24 h, followed by Western blot analysis of APP CTFs with the APP antibody. The membrane was reprobed with the P-T668 antibody to detect Thr668-phosphorylated β-CTF (P-β-CTF). (B) Band intensities in (A) were quantified by densitometric analysis, and the relative expression levels of β-CTF were calculated. Data are presented as means ± SEM from three independent experiments. (C) ELISA analysis of secreted Aβ40 from cells treated as in (A). Data are presented as means ± SEM from three independent experiments. $^*p < 0.05$, compared with control (paired *t* test).

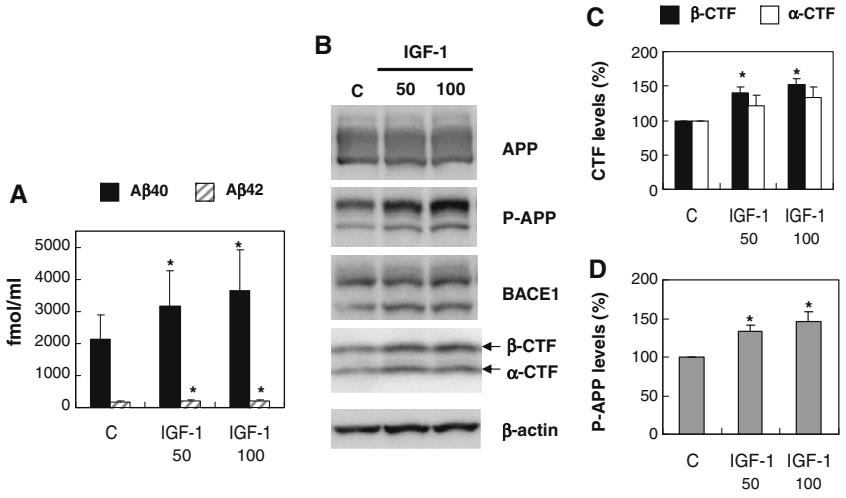

Fig. 1. Effects of IGF-1 on secreted Aβ and cellular levels of APP, P-APP, BACE1 and APP CTFs. (A) ELISA analysis of secreted Aβ40 and Aβ42 from SH-swAPP cells incubated without (Control: C) or with 50 or 100 ng/mL IGF-1 for 24 h. (B) Western blot analysis of lysates of cells treated as in (A). The membrane was probed with the antibodies against APP, P-APP, BACE1, and β-actin. For analysis of APP CTFs, proteins were separated on Tris/Tricine gels. (C and D) Band intensities in (B) were quantified by densitometric analysis, and the relative expression levels of β-CTF and α-CTF (C) and P-APP (D) were calculated. Data are presented as means ± SEM from three independent experiments. $^*p < 0.05$, compared with control (paired *t* test).

treatment also dose-dependently increased secretion of Aβ40 (Fig. 2C). Because this cell line secreted only small amounts of Aβ, Aβ42 levels were below the detection limits. Furthermore, Western blot analysis with the P-T668 antibody showed that levels of β-CTF with phosphorylation at Thr668 were also markedly increased by IGF-1 (Fig. 2A). These results suggest that the effect of IGF-1 on APP β-CTF levels does not depend on β-secretase processing of APP.

To determine whether the IGF-1-induced augmentation of APP-CTFs is dependent on γ-secretase processing, we treated SH-β-CTF cells with IGF-1 in the presence of the well-known γ-secretase inhibitors, DAPT and L-685,458. In cells treated with DAPT or L-685,458 alone, β-CTF levels were markedly elevated, and α-CTF appeared. Levels of β-CTF and α-CTF were even greater in cells treated with DAPT plus IGF-1 or with L-685,458 plus IGF-1, compared with cells treated with the respective inhibitor alone (Fig. 3A and B). In addition, the level of T668-phosphorylated β-CTF was also markedly increased in IGF-1-treated cells in the presence of γ-secretase inhibitors, compared with the control (Fig. 3A and B). These results suggest that IGF-1 promotes Aβ production through a γ-secretase-independent mechanism.

We further investigated the role of APP phosphorylation at Thr668 in the IGF-1-induced increase in the level of APP CTF and Aβ by utilizing SH-SY5Y cells expressing β-CTF with a Thr668 to alanine substitution. We confirmed by Western blot analysis that β-CTF levels were similar in cells expressing wild-type and mutant β-CTF, but that Thr668-phosphorylated β-CTF was not produced by cells expressing the mutant β-CTF (Fig. 4A). We found that IGF-1 treatment of the cells did not alter the level of β-CTF (Fig. 4B and C), suggesting that phosphorylation of APP at Thr668 plays a critical role in the IGF-1-induced increase in APP CTFs.

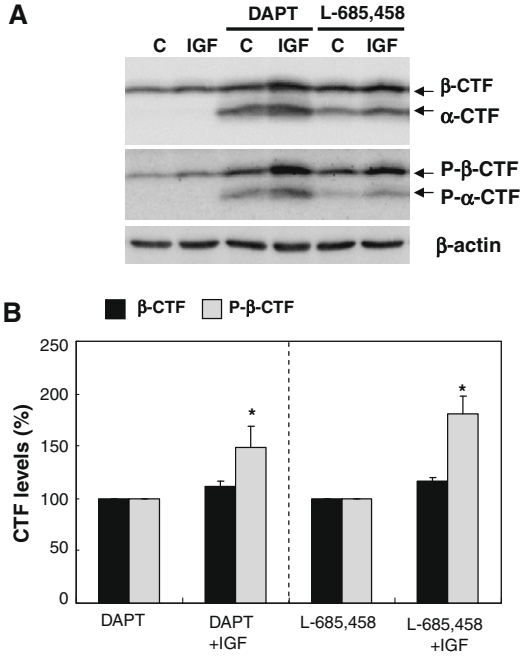

Fig. 3. IGF-1 increases β-CTF levels in the presence of γ-secretase inhibitors. (A) SH-β-CTF cells were pretreated with 0.5 μM DAPT or L-685,458 for 2 h, and then treated without or with 100 ng/mL IGF-1 in the presence of the γ-secretase inhibitors for 24 h. Cell lysates were subjected to Western blot analysis as described in Fig. 2. Thr668-phosphorylated β-CTF and α-CTF are referred to as P-β-CTF and P-α-CTF, respectively. (B) Band intensities in (A) were quantified by densitometric analysis, and the relative expression levels of β-CTF and P-β-CTF were calculated. Data are presented as means ± SEM from three independent experiments. $^{*}p < 0.05$, compared with control (paired *t* test).

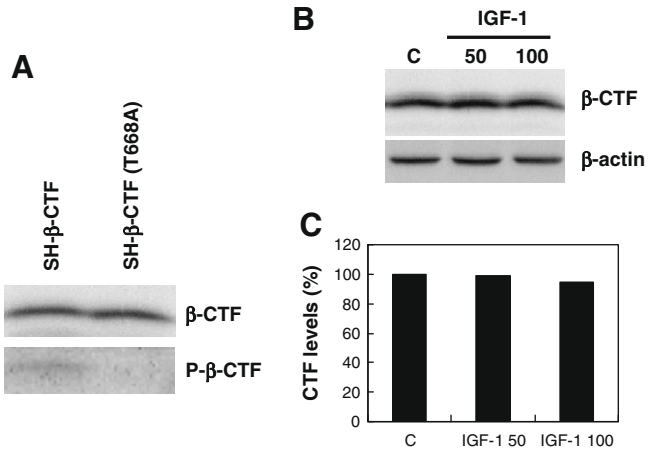

Fig. 4. IGF-1 does not increase β-CTF levels in cells expressing the Thr668Ala mutant β-CTF. (A) Cells expressing wild-type or Thr668Ala mutant β-CTF were treated with DAPT for 16 h, followed by Western blot analysis as described in Fig. 2. (B) Western blot analysis of lysates of Thr668Ala mutant β-CTF-expressing cells treated with indicated concentrations of IGF-1 for 24 h. (C) Band intensities in (B) were quantified by densitometric analysis, and the relative expression levels of β-CTF were calculated. Data are presented as means from duplicate experiments.

Discussion

In this study, we have demonstrated that IGF-1 promotes Aβ secretion by neuroblastoma SH-SY5Y cells expressing swAPP and by cells expressing APP β-CTF. This enhanced Aβ secretion was accompanied by increased levels of β-CTF in both cell types. IGF-1 increased β-CTF levels in the presence of γ-secretase inhibitors, suggesting that the effect was secretase-independent. Thus, it is plausible that IGF-1 induces an increase in the level of β-CTF by a secretase-independent mechanism, and that this increase in the availability of β-CTF, a γ-secretase substrate, leads to enhanced Aβ secretion. Furthermore, we found that IGF-1 increases levels of APP phosphorylation at Thr668 in both APP-expressing cells and β-CTF-expressing cells and that IGF-1 did not affect β-CTF levels in cells expressing the Thr668Ala mutant β-CTF. Together, these findings indicate that Thr668 phosphorylation plays a critical role in the IGF-1-induced increase in the level of β-CTF and Aβ secretion.

The mechanism underlying the IGF-1-induced increase in β-CTF most likely involves the phosphorylation of APP and β-CTF at Thr668. It is possible that β-CTF with Thr668 phosphorylation could be more resistant to degradation by non-secretase systems such as the proteasomal system [21], thereby resulting in an increase in the steady-state level of β-CTF. Alternatively, intracellular trafficking of phosphorylated β-CTF could be altered in such a way that less β-CTF is transported to non-secretase degradation machineries. Consistent with our findings, a previous study found that phosphorylated APP CTFs accumulated to a higher level than whole APP CTFs upon γ-secretase inhibition [22]. Further research is required to elucidate the precise mechanism of the modulation of APP CTF levels by phosphorylation.

A previous study found that IGF-1 induces an increase in β-secretase activity, the steady-state level of BACE1, and Aβ secretion in neurons after chronic treatment with IGF-1 [23]. Under our experimental conditions, in which a short 1 day treatment instead of chronic treatment with IGF-1 was used, IGF-1 did not alter the level of BACE1. However, in both experimental systems, IGF-1 signaling appeared to enhance Aβ production. Our data are not consistent with the findings of a previous study reporting that IGF-1 induces the secretion of sAPP and reduces Aβ production [24]. The reason for this discrepancy remains unknown, but it may be due to differences in cell culture conditions.

The signaling mechanism linking IGF-1 with APP phosphorylation remains unknown. Two major pathways, the phosphatidylinositol 3-kinase (PI3-K) and mitogen-activated protein kinase (MAPK) cascades, are known to be triggered by activation of IGF-1 receptors [5,25]. Previous studies have suggested that several kinases are involved in APP phosphorylation at Thr668, including cyclin-dependent kinase 5 (Cdk5) [26–28], c-Jun NH_2-terminal kinase [29,30], and dual-specificity tyrosine(Y)-phosphorylation regulated kinase 1A (DYRK1A) [31]. It is possible that one or more of these kinases are involved in the IGF-1-mediated phosphorylation of APP.

IGF-1 was reported to favor clearance of Aβ from the brain by enhancing the transport of the Aβ carrier proteins into the brain [32], and was also shown to have therapeutic efficacy in a mouse model of AD [33]. A clinical trial was recently conducted to test the efficacy of the drug MK-677, which stimulates growth hormone release and produces a robust increase in the serum level of IGF-1, but this drug was not effective in slowing the progression of AD [34]. Our findings suggest that IGF-1 induces an increase in the production of Aβ, which possibly diminishes its beneficial effect on the clearance of Aβ. Thus, the clinical application of IGF-1 or agents that stimulate IGF-1 signaling for the treatment of AD should be considered with great caution.

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