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Insulin inhibits Aβ production through modulation of APP processing in a cellular model of Alzheimer's disease

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Abstract

OBJECTIVE: Amyloid-beta (Aβ) is a 36–43 amino acid peptide that is derived by processing of the beta-amyloid precursor protein (APP). Aβ plays a central role in the development of Alzheimer's disease (AD). Although growing evidence suggests that insulin has important functions in Aβ metabolism, the underlying mechanisms are still unknown.
METHODS: Using an SH-SY5Y cell line overexpressing human APP Swedish mutant (APPsw), we evaluated the effect of insulin on APP processing and Aβ production by using western blot analysist.
RESULTS: Our data showed that administration of insulin reduced the Aβ generation in culture media with a concomitant decreases in the levels of β-secretase BACE1, secreted extracellular domain (sAPPβ) and a fragment of 99 amino acids (C99) in APPsw cells. We further showed that insulin increased the levels of α-secretase ADAM10, a secreted extracellular domain secreted (sAPPα) and a fragment of 83 amino acids (C83) in APPsw cells.
CONCLUSION: Our present data suggest that insulin could inhibit Aβ production through modulation of APP processing by increasing cleavage at the α-secretase site and decreased cleavage at the β-secretase sites.

INTRODUCTION

Alzheimer disease (AD) is a progressive neurodegenerative disease one of the widely accepted AD mechanisms is that the accumulation of senile plaques in the brain. Amyloid-β-protein (Aβ) is the main component of senile plaques which is derived from the beta-amyloid precursor protein (APP). APP has two metabolic pathways, namely the α-secretase pathway and the β-secretase pathway. Under physiological conditions, the majority of APP is cleaved by α-secretase into a secreted extracellular domain (sAPPα) and a fragment of 83 amino acids (C83), and sAPPα is further cleaved by γ-secretase into p3 peptide and the APP intracellular domain (AICD). The cleavage site of α-secretase prevents the generation of Aβ with a complete molecular sequence. A very small part of APP is cleaved by β-secretase and generates a secreted extracellular domain (sAPPβ) and a C-terminal membrane-bound fragment (C99), which is further cleaved by γ-secretase into Aβ (Zhang *et al.* 2007).

Recently, some studies have shown that the insulin in the periphery also crosses the blood-brain barrier (BBB) and plays important roles in the central nervous system (CNS), including metabolic, neurotrophic, neuromodulatory, and neuroendocrine actions. Furthermore, impaired insulin response has been linked to the occurrence of AD. Some clinical evidence suggests that administration of insulin and glucose enhances the memory of AD patients to a greater extent than injection of glucose alone (Beeri *et al.* 2008; Manning *et al.* 1993). Intranasal administration of insulin primarily improves hippocampus-dependent memory function (Reger *et al.* 2008; Schulingkamp *et al.* 2000). Some evidence has indicated that insulin regulates the metabolism of Aβ and tau, which are two proteins that represent the building blocks of amyloid plaques and neurofibrillary tangles (NFTs) (Gasparini *et al.* 2002). *In vitro* studies have shown that insulin reduces the phosphorylation of tau, enhances the binding of tau to microtubules, and promotes microtubule assembly through direct and reversible inhibition of glycogen synthase kinase-3 (GSK3β) in cultured human neurons and human neuroblastoma cells (Hong *et al.* 1997; Lesort *et al.* 1998; Lesort *et al.* 2000). However, the effect of insulin on the Aβ production is not clear *in vitro*. It has been shown that insulin may reduces Aβ40 and Aβ42 intracellular accumulation through non-amyloidogenic APP-processing pathway (Pandini *et al.* 2013). However, the results of Qiu et al have shown that insulin increases the extracellular concentration of Aβ, since insulin competes with Aβ for insulin-degrading enzyme (IDE) (Qiu *et al.* 1998). Gasparini et al showed that the insulin directly increases Aβ secretion and decreases the intracellular levels of Aβ peptides by stimulating their intracellular trafficking in neuronal cultures (Gasparini *et al.* 2001). Moreover, a previous study found that insulin bound to its cognate receptor and affected APP processing and subsequent production of Aβ in a streptozotocin (STZ)-induced diabetic AD mouse model (Wang *et al.* 2011). However, whether insulin is involved in APP processing and Aβ metabolism in the same way remained largely unknown *in vitro*.

As APP and its processing secretases are all integral membrane proteins, we developed an SH-SY5Y cell line overexpressing human APP Swedish mutant (named "APPsw cells" for short) in this study. Using this system, we analyzed whether insulin influence amyloidogenic and nonamyloidogenic processing, to further clarify the role of insulin on Aβ generation *in vitro*.

METHODS

Cell cultures

SH-SY5Y human neuroblastoma cells transfected with APPsw were cultured in DMEM/F12 supplemented with 10% heat-inactivated fetal calf serum, 500 μg/ml G418, 100 IU/ml penicillin, and 100 g/ml streptomycin at 37 °C in humidified 5% CO_2 air. At the 2nd day after seeding, the medium was changed to serum-free medium 2 h before insulin treatments. Cells were then treated with 0, 10, 100, or 1000 nM insulin from bovine pancreas (Sigma) for 12 h in 4 ml of serum-free culture medium.

Western blot analysis

Media were collected, centrifuged briefly to remove cell debris, and sequentially immunoprecipitated first for sAPPα and then sAPPβ. Cells were scraped from plates in ice-cold phosphate-buffered saline (PBS) with a rubber policeman. After centrifugation the pellets were homogenized in lysis buffer containing 150 mM sodium chloride, 50 mM Tris-hydrochloride, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% SDS, 1 mM phenylme-thylsulfonyl fluoride (PMSF), 10 mg/ml leupeptin, 1 mM Na_3VO_4, and 1 mM NaF, and then incubated for 2 h at 4°C. The homogenate was centrifuged at 12,000 rpm for 30 min and the supernatant was divided into aliquots and frozen at −80 °C. The total protein extract (40 μg) was separated on SDS-polyacrylamide gels and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were then incubated overnight at 4 °C in the following specific primary antibodies: rabbit anti-C-terminal fragments of APP (CTFs, 1:1000, Sigma-Aldrich), mouse anti-sAPPα (1:500, Immuno-Biological Laboratories), mouse anti-sAPPβ (1:500, Immuno-Biological Laboratories), rabbit anti-ADAM10 (1:1000, Millipore), rabbit anti-BACE1 (1:1000, Sigma), rat anti-PS1 (1:500, Millipore), and mouse β-actin (1:5000, Santa Cruz Biotechnology).

After washing with Tris-buffered saline-Tween (TBST), the membranes were incubated with horseradish peroxide-conjugated second antibody (1:5000, Santa Cruz Biotechnology) for 1 h at room temperature. Immunoreactive bands were visualized using the Super Signal West Pico Chemi-luminescent Substrate (Pierce Biotechnology, Rockford, IL) using Chem Doc XRS with Quantity One software (BioRad, USA). The bands were scanned and the intensities of the bands were measured using Image-pro Plus 6.0 analysis software.

Sandwich ELISA

The cell culture media of APPsw SH-SY5Y cells were collected. The cell media were centrifuged at 2000 rpm for 5 minutes to precipitate cells in the media. The concentration of Aβ40 and Aβ42 were measured using an ELISA kit (Invitrogen) according to the manufacturer's instruction. The absorbance was recorded at 450 nm using a 96-well plate reader.

Statistical analysis

All values are expressed as mean±standard deviation (SD). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by a two-tailed student's *t*-test. All data were analyzed using SPSS software (IBM, NY, USA), and $p<0.05$ was considered statistically significant.

RESULTS

Effects of insulin on the protein expression of APP cleavage enzymes

In order to analyze whether insulin affect APP cleavage by modulating protein expression of APP cleavage enzymes involved in the nonamyloidogenic and amyloidogenic processing of APP, we performed western blot analysis to examined the levels of ADAM10, BACE1, and PS1 (Figure 1A). APPsw cells treated with 100 nM and 1000 nM insulin showed an increased level of ADAM10, by 160.44±14.36% and 152.25±12.14%, respectively ($p<0.05$; Figure 1B), and a reduced level of BACE1, by 46.68±14.19% and 40.13±13.26%, respectively ($p<0.01$; Figure 1C) in APPsw cells. No statistically significant changes in the protein levels of PS1 were detected between insulin-treated and control cells (Figure 1D).

Effects of insulin on APP fragments in the APPsw SH-SY5Y cells

APPsw cells were treated with serum-free DMEM/F12 alone or with increasing concentrations of insulin (10 nM, 100 nM, or 1000 nM). First, we measured the protein expression of full APP as well as APP C-terminal fragments C83 and C99 using rabbit anti-CTFs antibody in cells (Figure 2A). Immunoblots revealed that there were no changes in the expression levels of any of the APP proteins ($p>0.05$; Figure 2B). We also observed two discrete bands corresponding to the APP C-terminal fragments, C99 and C83 (Figure 2A). Western blot analysis showed significant reduction of C99 expression at 100 and 1000 nM of insulin (69.09±11.46% and 65.32±10.37%, respectively; $p<0.05$; Figure 2C). Insulin treatment at 1000 nM concentration increased the levels of C83 levels in cells by 134.65±12.34% ($p<0.05$; Figure 2D). In addition, we examined the products of α- and β-secretase-mediated APP cleavage (sAPPα and sAPPβ) in media (Figure 2E). The levels of sAPPα in medium were increased by 172.18±15.74% and 195.49±18.7% at 100 and 1000 nM, respectively ($p<0.05$ and $p<0.01$; Figure 2F). Insulin treatment at 100 and 1000 nM concentrations significantly decreased the level of sAPPβ by 53.15±11.08% and 56.67±9.26%, respectively ($p<0.05$ and $p<0.01$; Figure 2G).

Effects of insulin on Aβ40 and Aβ42 in cell culture medium

To determine whether insuoin affects Aβ generation, APPsw cells were treated with different concentrations of insulin and the levels of Aβ40 and Aβ42 were examined by ELISA. Aβ ELISA showed that 100 nM and 1000 nM concentrations insulin significantly decreased the level of Aβ40 and Aβ42 in cell culture medium

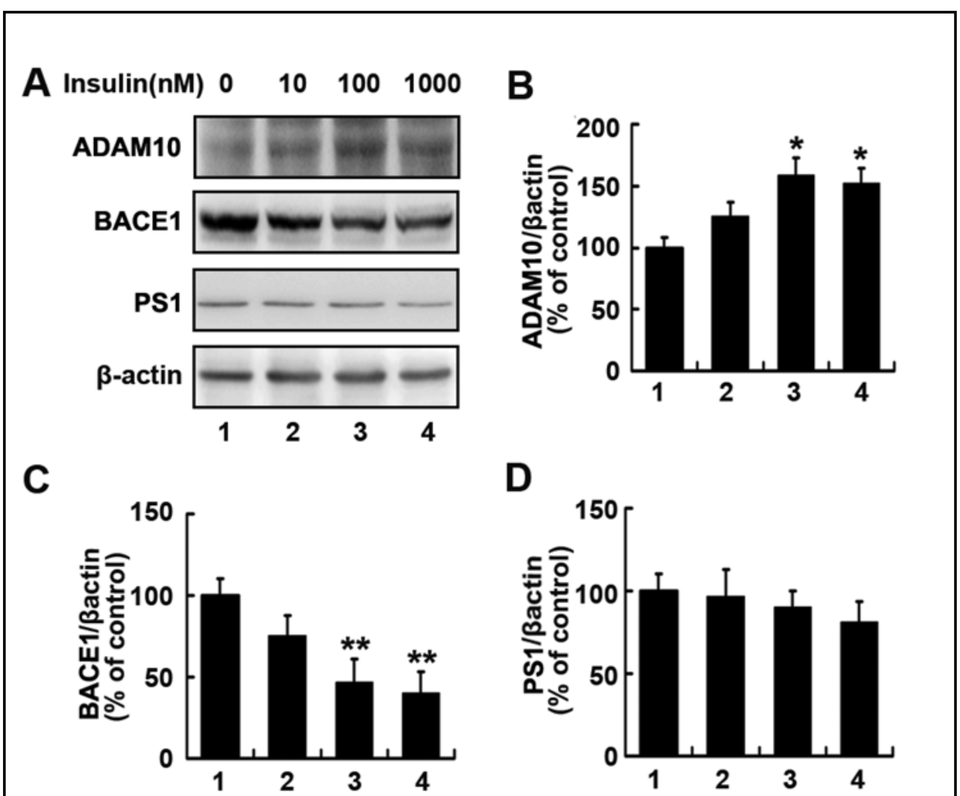

Fig. 1. Insulin affects APP cleavage enzymes in APPsw SH-SY5Y human neuroblastoma cells. APPsw SH-SY5Y cells were incubated in the absence (lane 1) or presence of 10 nM (lane 2), 100 nM (lane 3), or 1000 nM (lane 4) insulin for 24 h. Immunoblots show the expression levels of ADAM10, BACE1 and PS1 in the APPsw SH-SY5Y cells. β-actin was used as a loading control (A). Quantification of the protein expression of ADAM10 (B), BACE1 (C) and the PS1 (D) in insulin-treated SH-SY5Y APPsw cells. *$p<0.05$, **$p<0.01$.

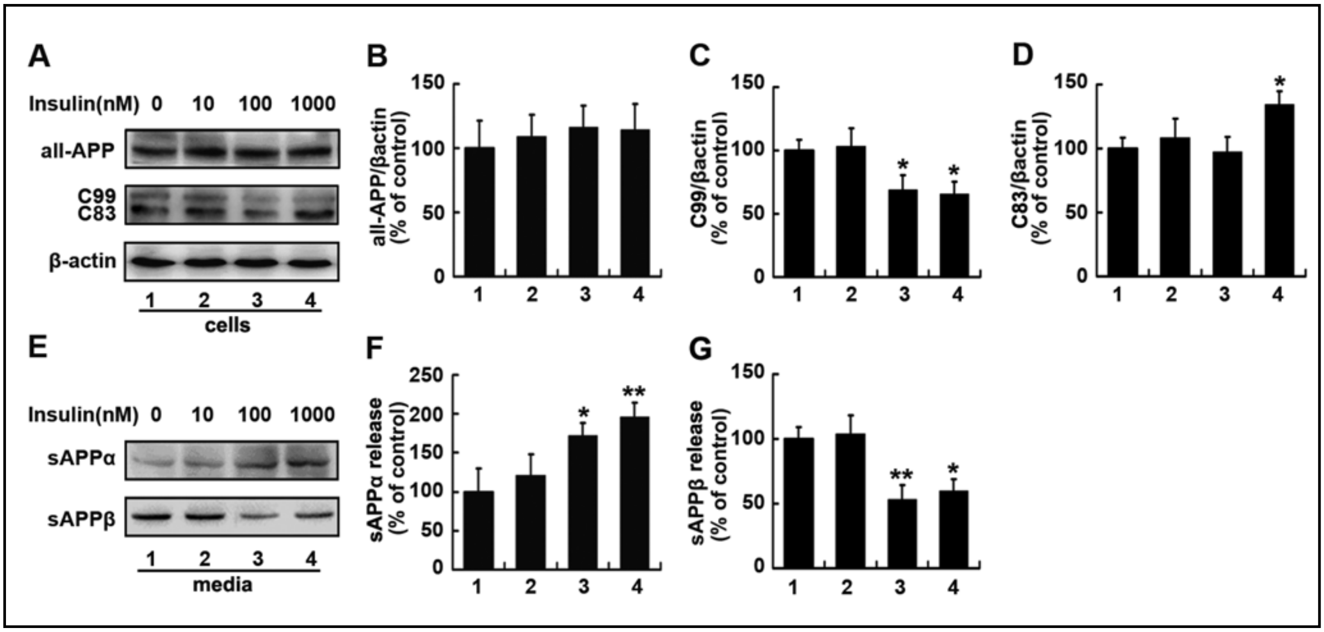

Fig. 2. Insulin affects APP fragments in APPsw SH-SY5Y human neuroblastoma cells. APPsw SH-SY5Y cells were incubated in the absence (lane 1) or presence of 10 nM (lane 2), 100 nM (lane 3), or 1000 nM (lane 4) insulin for 24 h. Immunoblots show the expression levels of full APP and CTFs in the APPsw SH-SY5Y cells. β-actin was used as a loading control (A). Quantification of the expression of all-APP (B), the C99 levels (C) and the C83 levels (D) in insulin-treated SH-SY5Y APPsw cells. Immunoblots show the expression levels of sAPPα and sAPPβ in medium (E). Quantification of the release of sAPPα levels (F) and sAPPβ levels (G) in the conditioned media. *$p<0.05$, **$p<0.01$.

when compared with the control (Figure 3). The cells cultured under 100 nM concentrations insulin for 24 h, where the Aβ40 and Aβ42 levels in cell culture medium were reduced by 65.11±9.25% and 55.95±13.87%, respectively ($p<0.01$; Figure 3A). The Aβ40 and Aβ42 levels in medium were decreased by 48.97±110.35% and 40.34±9.36% at 1000 nM concentrations insulin, respectively ($p<0.05$ and $p<0.01$; Figure 3B).

DISCUSSION

AD is a progressive neurodegenerative disease clinically characterized by progressive cognitive impairment and pathologically characterized by the presence of extracellular senile plaques and intracellular neurofibrillary tangles (NFTs) in the brain. Senile plaques are largely composed of Aβ, and the deposition of Aβ and subsequent formation of senile plaques are thought to be the primary cause of AD. Aβ is a product that results from the cleavage of its precursor protein (APP), a ubiquitous single pass trans-membrane protein. APP undergoes two major pathways to product Aβ, one non-amyloidogenic and one amyloidogenic. In the amyloidogenic pathway, APP is first hydrolyzed by β-Site APP cleaving protein 1 (BACE1), a membrane-bound aspartyl-protease, generating sAPPβ and C99. γ-secretase further cleaves C99 to release AICD and the Aβ which aggregates to form amyloid plaques in the brain. The γ-secretase has been identified as a multimeric complex of at least four transmembrane proteins, presenilin 1 (PS1) or presenilin 2 (PS2), nicastrin, anterior pharynx-defective 1 (Aph-1), and presenilin enhancer 2 (Pen-2). In the non-amyloidogenic pathway, APP is cleaved by α-secretase and releases sAPPα and C83. A disintegrin and metalloproteinase 10 (ADAM10) is a major α-secretase involved in non-amyloidogenic processing of the amyloid precursor protein. γ-secretase cleaves C83 to produce p3 and AICD, both of which are degraded rapidly (Rothhaar *et al.* 2012).

A few longitudinal studies have reported that insulin not only controlls systemic blood glucose concentrations but also contributes to several neurobiological processes in particular energy homeostasis and cognition. Recently, considerable evidence suggests that insulin as an important neuromodulator has a direct effect on AD (Pavlik *et al.* 2013; Gasparini *et al.* 2003). Some evidence indicates that insulin involves in the metabolism and clearance of Aβ. It has been shown that insulin inhibits Aβ breakdown through the IDE, one of the main proteases involved in Aβ degradation (Gasparini *et al.* 2001; Bossy *et al.* 2008). However, the effect of insulin on Aβ metabolism is far more complex and the mechanism has not been fully elucidated.

In the present study, we found that the insulin inhibited Aβ production in APPsw cells by Elisa. We also showed that a certain concentration of insulin in the cultured APPs cells was associated with alterations to APP processing involving increased cleavage at the α-secretase ADAM10 site and decreased cleavage at the β-secretase BACE1 sites. Our data, using a SH-SY5Y cell line overexpressing human APPsw, demonstrates a previously unknown mechanism by which insulin regulates Aβ generation, through modulation of APP processing.

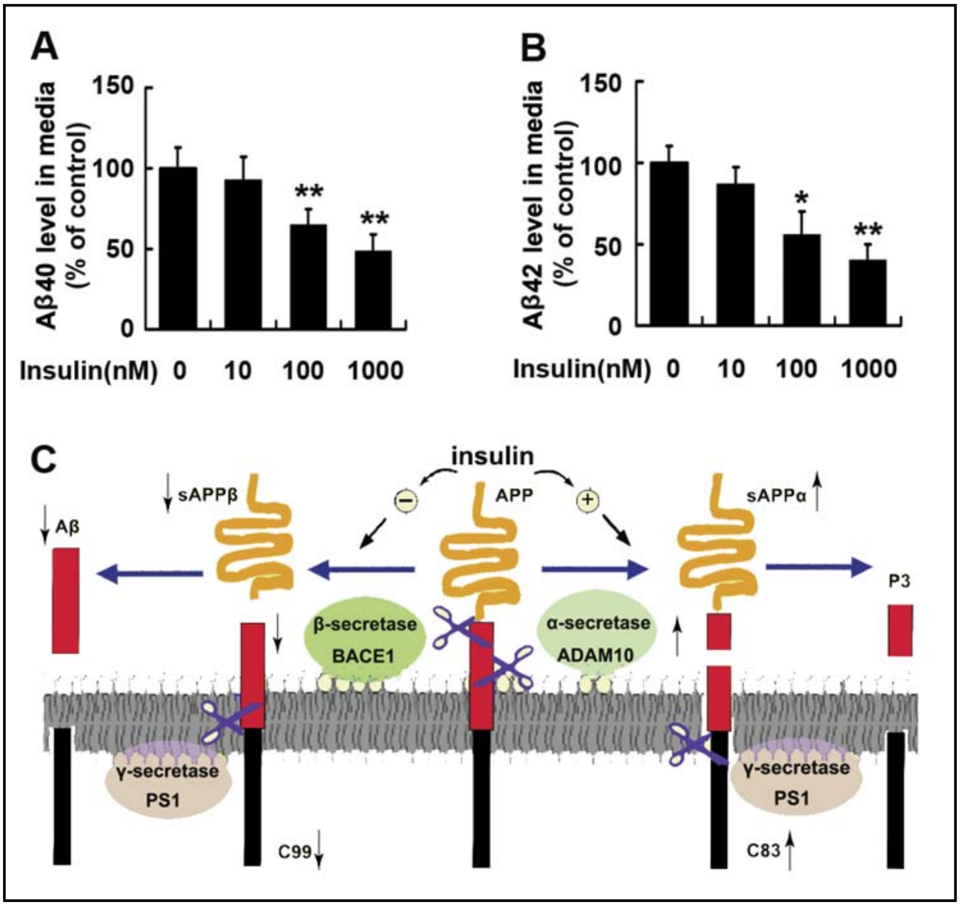

Fig. 3. Insulin significantly decreased Aβ in APPsw SH-SY5Y human neuroblastoma cell culture media. APPsw SH-SY5Y cells were incubated in the absence or presence of 10 nM, 100 nM, or 1000 nM insulin for 24 h. Conditioned medium was collected and analyzed by sandwich Aβ ELISA to measure Aβ40 and Aβ42 levels. Quantification of the Aβ40 levels (A) and Aβ42 levels (B) in the conditioned media. Proposed model of metabolism of the insulin- modulated APP processing (C). *$p<0.05$, **$p<0.01$.

To study the possible mechanism of how insulin decreased the Aβ level, we used western blot analysis to detect the protein expression of α-secretase ADAM10 and α-secretase-generated sAPPα and C83 fragments. Higher α-secretase activity is associated with a increased production of sAPPα and C83 (Vagnoni *et al.* 2012). Our results showed that the protein levels of ADAM10 and C83 in APPsw cells were significantly at 100 and 1000 nM of insulin. In addition, the secreted sAPPα in cultured media levels is also increased in the same condition. Importantly, the results are consistent with findings by Solano et al that showed that human recombinant insulin can increase sAPPα release from SH-SY5Y cells (Solano *et al.* 2000). In contrast to Aβ, sAPPα is a product of the non-amyloidogenic cleavage pathway of APP processing and has been previously shown to have several neuroprotective functions (Hartl *et al.* 2013). Therefore, we speculated that insulin promote the activity of α-secretase, which leads to a lower metabolism of APP through the β-secretase pathway and an decrease in Aβ production. Indeed, besides these changes in the non-amyloidogenic cleavage pathway of APP processing, insulin reduces the levels of BACE1 and BACE1-derived APP cleavage fragments, including sAPPβ and C99. APP is cleaved to form neurotoxic Aβ and is involved in the pathogenesis of AD through the β-secretase pathway (Hampel *et al.* 2009). Thus, reduced BACE1 levels and a subsequent decrease in the BACE1-mediated cleavage fragments appear to indicate that insulin could reduce amyloidogenesis and the risk of developing AD. Together, our results indicate that insulin leads to a shift in APP processing by increasing cleavage at the α-secretase site and decreased cleavage at the β-secretase sites (Figure 3C). The findings clearly point to insulin may be envisioned as an alternative strategy in developing AD therapeutics.

In conclusion, our current study indicates that insulin under certein concentration inhibits Aβ production through APP processing in human neuroblastoma cells overexpressing. These findings should be considered in the future development of therapeutic strategies for AD.

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