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EXPERIMENTAL ARTICLES

Effects of Rivastigmine on Secreted Amyloid Precursor Protein and Beta-Amyloid Secretion in Neuroblastoma SK-N-SH Cells[1]

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Abstract—Alzheimer's disease (AD) is a neurodegenerative disorder characterized clinically by progressive impairment of memory and cognition. The currently available pharmacological treatment of AD consists mainly of cholinesterase inhibitors. Rivastigmine is one of the cholinesterase inhibitors clinically used to treat this disease, and many clinical trials have indicated that it did alleviate some AD symptoms without causing apparent side effects. Since the amyloid precursor protein (APP) processing imbalance plays a crucial role in AD pathogenesis, the effects of rivastigmine on APP processing were investigated. In neuroblastoma SK-N-SH cells, rivastigmine significantly increased the secretion of sAPPα and decreased the release of Aβ40 and Aβ42 as compared with control group, but it has no effect on cellular full length APP expression. Rivastigmine significantly increased α-secretase activity and decreased β-secretase activity as compared with control group. The increased sAPPα can be partially blocked by muscarinic receptor inhibitor scopolamine but not by nicotinic receptor antagonist α-Bungarotoxin. The effect of rivastigmine on sAPPα can be partially reversed by PKC inhibitor GF109203X, ERK inhibitor PD98059 and JNK inhibitor SP600125. The data present here indicated that rivastigmine can regulate APP processing in vitro by increasing sAPPα secretion and decreasing Aβ release and this pharmacological property may underlie the clinical effect of the drug in the treatment of AD patients.

Keywords: Alzheimer's disease, cholinesterase inhibitor, rivastigmine, amyloid precursor protein, beta amyloid

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive impairment of memory and cognition, and is the most frequent form of dementia found in the elderly. One of the hallmarks of AD is the presence of senile plaques in the hippocampus, which are primarily formed from the extracellular deposition of beta amyloid (Aβ), a 40–42(3) amino acids polypeptide [1]. This 4-kDa protein is generated via proteolytic cleavage of the amyloid precursor protein (APP). APP can be processed by two alternative, mutually exclusive post-translational pathways: amyloidogenic and non-amyloidogenic. In the amyloidogenic pathway, APP is first cleaved by β-secretase at the amino-terminal side of the Aβ sequence to generate a soluble peptide sAPPβ and an intracellular carboxyl-terminal fragment, C99. C99 is then cleaved by γ-secretase to release Aβ. In the non-amyloidogenic pathway, APP is hydrolyzed by α-secretase within the Aβ sequence to produce a soluble sAPPα and a transmembrane fragment C83. As α-secretase hydrolyzes APP at Lys16-Leu17 within the Aβ sequence, the non-amyloidogenic pathway not only precludes the formation of Aβ, but also generates a neurotrophic sAPPα. Since α-secretase and β-secretase compete for the same substrate APP, theoretically, there would be more sAPPα secretion and less Aβ production if more APP were processed by α-secretase [2, 3]. It has documented on numerous occasions that Aβ can aggregate and form deposits that finally lead to neuronal dysfunction [4]. Thus decreasing secretion of Aβ from its precursor APP should be very favorable to AD prognosis. As the detailed pathogenesis of AD is still not clear, the treatment at present is limited to drugs that could alleviate the symptoms of AD. Currently available drug therapies for AD consist primarily of cholinesterase inhibitors. The reason is based upon the fact that the cholinergic transmission is attenuated and the cholinergic neurons are progressively degenerated in AD patients.

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It has been reported that stimulation of M1 and M3 muscarinic receptors increased the release of sAPPα and decreased the secretion of Aβ [5]. Since then the effects of "cholinergic effect" of some cholinesterase inhibitors on APP metabolism have been extensively investigated. Shaw reported that phenserine could decrease the expression of APP, the secretion of sAPPα and Aβ release [6]. Treatment of SH-SY5Y cells with metrifonate and dichlorvos increased the production of sAPPα without affecting cellular APP expression [7]. Huperzine A increased sAPPα release in human embryonic kidney 293 Swedish mutant cells [8]. Recent work in our team showed that PMS777, a new cholinesterase inhibitor with anti-platelet activated factor activity, could decrease sAPPα secretion and Aβ42 release without affecting full-length APP expression [9]. Zimmermann indicated that donepezil could decrease Aβ secretion and increase sAPPα release [10]. The data implied that although all these cholinesterase inhibitors could delay the degradation of acetylcholine and thus compensate the attenuated cholinergic transmission in the synapse, their effects on APP processing were different. That is, their effects on APP processing may beyond those mediated by the cholinergic system.

Rivastigmine is a dual acetyl- and butyrylcholinesterase inhibitor approved by the FDA for the treatment of AD. Clinical trials have indicated that it did alleviate some of the cognitive and noncognitive symptoms of AD patients [11, 12]. A recent study showed that rivastigmine increased sAPPα secretion and decreased Aβ release [13]. Although this effect mirrors synaptic protein, the detailed mechanism is unclear. As APP processing represents a crucial step in the pathogenesis in AD development, we further investigated the detailed mechanisms that underline after rivastigmine administration. We investigated the effect of rivastigmine on some of the pathogenetic pathways leading to neurodegeneration in AD, i.e. APP expression and Aβ secretion, and its effects on α- and β-secretase activities. The involvement of cholinergic activity and the possible signaling pathways which regulating APP processing were also investigated here.

MATERIALS AND METHODS

2.1. Reagents

Rivastigmine was provided by Novartis Ltd (China). Dimethyl sulphoxide (DMSO), phenyl methanesulfonylfluoride (PMSF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-te-trazolium bromide (MTT), scopolamine, α-Bungarotoxin and mouse β-actin monoclonal antibody were provided by Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA). Mouse Alzheimer precursor protein monoclonal antibodies (22C11 and 6E10) were obtained from Chemicon International, Inc. (Temecula, CA). Protein kinase C (PKC) inhibitor GF109203X, extracellular signal regulated kinase (ERK) inhibitor PD98059 and c-Jun N-terminal kinase (JNK) inhibitor SP600125 were provided by Calbiochem (San Diego, CA). All other reagents were the highest grade available from Sigma-Aldrich unless otherwise indicated.

2.2. Cell Culture and Drug Treatment

Human neuroblastoma SK-N-SH cells were plated in 100 mm culture dishes (Corning Incorporated, Corning, NY) in DMEM containing 10% FBS, 1% penicillin and 1% streptomycin. Cells were grown at 37°C in a humid, 5% CO_2 environment. Twelve hours before drug treatment, the media was replaced with FBS-free media. Cells were then treated with rivastigmine (dissolved in distilled water) for 2 hours. Some cultures were also pretreated with various kinase inhibitors 30 minutes before rivastigmine challenge. Two hours after drug challenge, the conditioned media was collected, concentrated with Centricon YM-30 filter units (Millipore, Bedford, MA) and frozen at −20°C until further analysis. Cells were washed twice with cold phosphate buffered saline and lysed on ice with RIPA lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS), supplemented with 2 mM PMSF). After centrifugation at 12000 rpm for 30 minutes at 4°C, the supernatants were transferred to new microtubes and stored at −20°C. The protein concentration of each sample was quantified using the BCA assay (Pierce Biotechnology, Rockford, IL).

2.3. Western Blotting Analysis

Twenty micrograms of protein mixed with 5× loading buffer (0.313 M Tris-HCl (pH 6.8) at 25°C, 10% SDS, 0.05% bromophenol blue, 50% glycerol) and 20× reducing agent (2 M DTT) (Fermentas, Hanover, MD) was boiled for 5 min and loaded onto a 7.5% SDS-polyacrylamide electrophoresis gel(PAGE). After electrophoresis, the protein was electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membranes were saturated with 5% non-fat milk and incubated with primary antibodies (1 : 3000 for 6E10, 1 : 2500 for 22C11, and 1 : 10000 for β-actin) at 4°C overnight. After being washed 30 min in tris-buffered saline (TBS) with gentle agitation, the membranes were incubated with biotinylated anti-mouse/rabbit IgG secondary antibodies (Vector Laboratories, Burlingame, CA) in non-fat milk at room temperature for 1 h. The membranes were challenged with ABC agent (Vector Laboratories). After washing, the signals were developed with ECL advanced Western Blotting Detection kit (Amersham, UK). Band intensities were quantified by densitometric analyses using an Axio-

Cam digital camera (ZEISS, Germany) and the KS400 photo anaylsis system (Ver 3.0).

2.4. Enzyme-Linked Immunosorbent Assay (ELISA)

For the measurement of Aβ released into the media from vehicle and rivastigmine treated SK-N-SH cells, an ELISA kit was used. Briefly, following drug treatment, cell media was collected, treated with protease inhibitor cocktail and stored at −70°C for future use. Before detection, the samples were lyophilized at −160°C for 20 hours and resuspended in the sample buffer. Samples were detected as indicated in the manufacturer's instructions. A standard curve was performed in duplicate and the release of Aβ was evaluated using the standard curve as reference. The obtained values were then referred in terms of the total amount of proteins measured per well of lyophilized medium. The data were expressed as pg/mL and normalized to total proteins of cells per well in mg.

2.5. MTT Assays

Cell viability was evaluated with MTT assays. Briefly, 24 hours after plating 2×10^4 cells in each well of a 96 well plate, the original media was replaced with media containing MTT at a final concentration of 0.5 g/L. Four hours later, the media was discarded and DMSO was added for the colorimetric assay. Absorption was determined in a Tecan Sunrise Eliza-Reader (Switzerland) at $\lambda = 570/630$ nm after automatic subtraction of background signals. The results were expressed as a percentage of normal cells.

2.6. α- and β-Secretase Activity Detection

Following treatment with rivastigmine for 2 h, cells were washed with cold PBS and incubated with extraction buffer for 10 minutes on ice. Equal amount of proteins were added to a 96-well plate, mixed with reaction buffer and substrate at room temperature in the dark with gentle agitation for 2 hours. The substrates used here were APP peptides YEVHHQKLV and REEVNLDAEFKR for α- and β-secretase respectively using EDANS/DABCYL as the reporter system (provided by the manufacture). Signals were detected in a Tecan Sunrise Eliza-Reader (Switzerland) at an excitation wavelength of 350 nm and an emission wavelength of 500 nm after automatic subtraction of background signals. The results were expressed as a percentage of control groups.

2.7. Statistical Analysis

Quantitative analysis of Western blots was performed by calculating the relative density of the immunoreactive bands and expressed as a percentage of control values. Data were presented as the mean ± S.D. Each procedure was performed in duplicate in three to five independent experiments. Statistical analyses were carried out using one-way ANOVA, followed by a two-tailed Student's *t*-test. Multiple comparison tests were used when appropriate, and $P < 0.05$ was regarded as statistically significant.

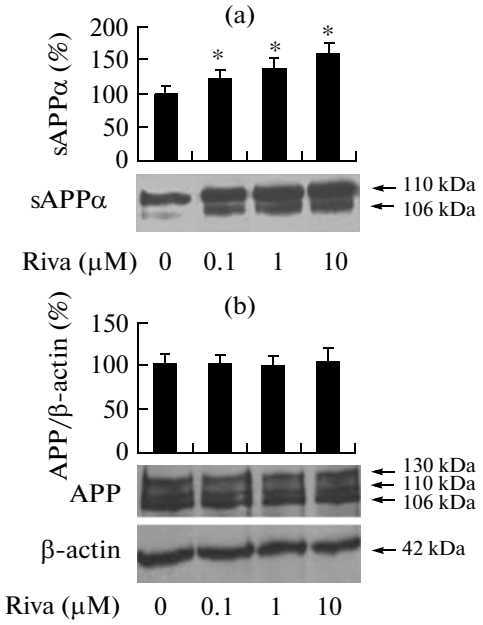

Fig. 1. The effect of rivastigmine on sAPPα secretion and cellular fulllength APP expression. (A) SK-N-SH cells were treated with vehicle or different concentrations of rivastigmine for 2 h and conditioned media was subjected to Western blot for sAPPα detection using 6E10 antibody. Densiometric values are expressed as a percent of the control, and represent the mean ± S.D. of three independent experiments (*$P < 0.01$ versus control). (B) After rivastigmine treatment, 20 μg of protein from cell homogenate was loaded onto SDS-PAGE for detection of cellular full length APP (using 22C11 antibody) and β-actin expression. Three independent experiments were performed in duplicate.

3. RESULTS

3.1. Rivastigmine Increased sAPPα Secretion

To detect sAPPα secretion from human SK-N-SH cells, 6E10 antibody was used. This antibody recognizes the 1–16 amino acid sequence of human Aβ, a region absent from both β-secretase processed APP and the amyloid precursor-like protein 2(APLP2). Thus, 6E10 detects sAPPα that specifically derives from non-amyloidogenic, α-cleavage. The results here indicated that rivastigmine at concentration 0.1 to 10 μM promoted sAPPα secretion into the cell media in human SK-N-SH cells as compared with the control group ($F = 96.356$, $P < 0.01$). Specific immunoreactive bands at 106 and 110 kDa, increased by rivastigmine treatment, were visible. The maximal effect of rivastigmine was seen at 10 μM, when rivastigmine increased sAPPα secretion approximately 1.5-fold over the control group (Fig. 1a). Cells were also treated with rivastigmine at different time

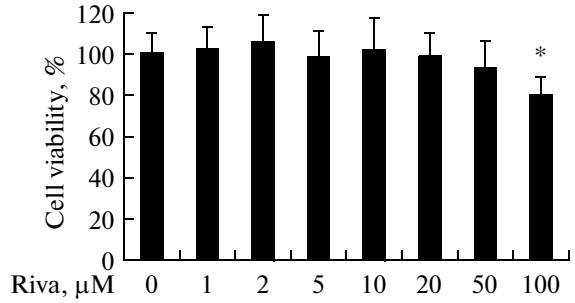

Fig. 2. Effect of rivastigmine on cell viability. SK-N-SH cells were treated with the indicated concentrations (0.1–100 μM) of rivastigmine for 24 hours. MTT assays were carried out to detect cell survial in a Tecan Sunrise Eliza-Reader at λ = 570/630 nm after automatic subtraction of background signals. Results are expressed as a percentage of normal cells. The data shown are representative of five independent experiments (*$P < 0.05$, versus control).

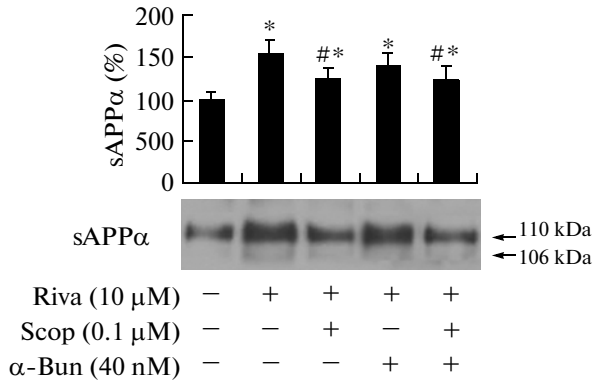

Fig. 3. Effect of cholinergic receptor inhibitors on sAPPα secretion. SK-N-SH cells were preincubated 40 min with either 100 nM scopolamine or 40 nM α-Bungarotoxin or both, and then treated with 10 μM rivastigmine for 2 h. The proteins released into the cell media were subjected to Western blot analysis for sAPPα secretion detection. Three independent experiments were performed in duplicate (*$P < 0.01$ versus control; #$P < 0.01$ versus 10 μM rivastigmine).

intervals and it was found that rivastigmine increased sAPPα secretion most at 2 h after rivastigmine challenge (data not shown). So all experiments were carried out at a 2 h treatment style.

To determine whether the promotion of sAPPα release by rivastigmine was the result of an increase in cellular APP expression, the 22C11 antibody, which recognizes the N-terminal of APP, was used to detect full length APP in cell homogenates. Using β-actin as an internal control, it showed that rivastigmine did not significantly influence cellular APP (molecular weight of 106, 110 and 130 kDa, Fig. 1b) expression ($F = 3.208$, $P > 0.05$). It was thus concluded that the promotion of sAPPα secretion by rivastigmine was possibly due to a shift in APP metabolism toward the α-secretase processing pathway.

3.2. Rivastigmine Decreased Aβ Release

Deposition of Aβ extracellularly is one of the hallmarks of AD pathology, and decreasing Aβ is one of the targets of AD therapy. Thus the effect of rivastigmine on Aβ release was investigated. ELISA data showed that rivastigmine significantly decreased Aβ40 [(35.24 ± 7.35) vs. (20.06 ± 5.48); $t = 10.325$, $P = 0.006$] and Aβ42 [(5.28 ± 1.35) vs. (3.25 ± 1.03); $t = 7.516$, $P = 0.018$] as compared with respective control group.

3.3. Rivastigmine Didn't Decrease Cell Viability

To exclude the possibility that the effect of rivastigmine on Aβ release was caused by its neurotoxicity on cells, MTT assays was used to determine the effect of rivastigmine on cell survival. The results in Fig. 2 revealed that 100 μM rivastigmine decreased cell numbers while 10 μM rivastigmine did not significantly decrease cell viability in SK-N-SH cells. This implied that the decreased Aβ release after 10 μM rivastigmine treatment was not induced by its neurotoxicity to cells, but might be mediated through its pharmacological properties.

3.4. The Effect of Rivastigmine on α- and β-Secretases Activity

To further explore the effect of rivastigmine on APP processing, its effect on α- and β-secretases activity was detected directly. The data here indicated that rivastigmine increased α-secretases activity 28.4 ± 10.5% as compared with control group ($t = 10.542$, $P = 0.008$). In accordance with ELISA experiments, rivastigmine decreased β-secretase activity 22.7 ± 12.2% compared with the control group ($t = 9.684$, $P = 0.025$).

3.5. Involvement of Cholinergic Effect in Regulating APP Processing by Rivastigmine

To explore whether rivastigmine modulated APP processing through cholinergic receptor dependent mechanism, SK-N-SH cells were first exposed to 100 nM scopolamine for 40 min in order to block the muscarinic receptors and then rivastigmine was added to the cell media for 2 h to investigate its effect on sAPPα release. As shown in Fig. 3, a significant increase in the release of sAPPα from rivastigmine treated cells was repeatedly found, but this effect was partially antagonized by pretreatment of cells with scopolamine. This implied that the effect of rivastigmine on sAPPα release may be mediated through cholinergic receptor. The cells were then exposed to 40 nM α-Bungarotoxin, a highly potent inhibitor of nicotinic receptor. The results showed that although α-Bungarotoxin slightly decreased sAPPα release as compared with rivastigmine-treated cells, this effect didn't reach statistical significance. Pretreated cells with both scopolamine and α-Bungarotoxin significantly blocked rivastigmine induced sAPPα secretion,

but failed to restore the secretion of sAPPα to vehicle level ($F = 29.853$, $P < 0.05$, Fig. 3).

3.6. The Signaling Pathways Involved in Rivastigmine-Induced sAPPα Secretion

To further explore the signal transduction pathways involved in the effect of rivastigmine on APP processing, several signal transduction inhibitors were added 30 min prior to the addition of 10 μM rivastigmine. The data showed that the stimulation of sAPPα secretion by rivastigmine could be partially alleviated by the PKC inhibitor GF109203X, ERK inhibitor PD98059 and JNK inhibitor SP600125 ($F = 48.625$, $P < 0.05$). The observations suggest that sAPPa secretion is mediated, at least in part, through PKC, ERK and JNK-dependent pathways (Fig. 4).

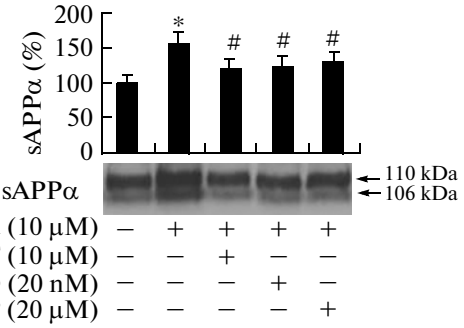

Fig. 4. Effect of various signal transduction pathways inhibitors on rivastigmine-induced sAPPα secretion. SK-N-SH cells were pretreated for 30 minutes with 10 μM GF109203X, 20 μM PD98059 or 20 μM SP600125, and then incubated with 10 μM rivastigmine for 2 h. Then the sAPPα secreted into the cell media was then detected. The experiment is a representative Western blot of three independent experiments (*$P < 0.01$ versus control; #$P < 0.01$ versus 10 μM rivastigmine).

4. DISCUSSION

Using human neuroblastoma SK-N-SH cells, we demonstrated here that rivastigmine can modulate APP processing by increasing sAPPα secretion and decreasing Aβ release. The reason that rivastigmine increase sAPPα secretion was not the result of increased synthesis of cellular total APP, as rivastigmine did not significantly influence total cellular APP expression. Nor rivastigmine decrease Aβ release was the neurotoxic effect of itself on cells, as rivastigmine didn't significantly affect cell viability. The increased secretion of sAPPα can be blocked by scopolamine pretreatment, suggesting involvement of cholinergic receptor in the regulation of APP metabolism. Also, the increased sAPPα secretion after rivastigmine may be mediated through PKC, ERK and JNK signaling pathways.

Because of the central role of APP processing in AD pathophysiology, extensive work has been performed to explore possible drug targets for AD therapy. Among them, an alternative strategy might be to stem APP processing toward the nonamyloidgenic pathway and the experimental data are mostly encouraging [2, 14, 15]. Epidemiological studies have shown that the patients who take statins, estrogen, testosterone and non-steroidal anti-inflammatory drugs (NSAIDs) have a lower risk of developing AD than the patients who do not take these drugs. Subsequent studies indicated that all of these drugs can regulate APP processing [16–19]. These results implied that drugs that can regulate APP shedding toward the α-secretase processing pathway or increase α-secretase activity may have therapeutic potential for AD.

The data here showed that rivastigmine increased sAPPα secretion, in other words, rivastigmine might increase nonamyloidgenic pathway activity, which was further supported by the increased α-secretase activity. The reason(s) why it acted to increase the production of sAPPα was not clear. It might increase the α-secretase activity by increasing α-secretase expression or its molecular maturation [20, 21], or it might increase the sensitivity of α-secretase to its substrate, APP. But the detailed mechanism of rivastigmine needs further research.

Another point was that rivastigmine also decrease Aβ release, the main components of senile plaques. And the β-secretase activity detection was in accordance with ELISA assay. Although decreasing Aβ secretion is very favorable for AD treatment, the detailed molecular mechanisms are to be elucidated. We speculated that rivastigmine might act to decrease β- and/or γ-secretase activity in an unknown way at present that finally decrease Aβ generation. We also can't exclude the possibility of the involvement of Aβ degrading enzyme [22] in rivastigmine's effect on Aβ release.

Nitsch and coworkers originally reported that the release of sAPPα may be stimulated by an activation of muscarinic M1 and M3 receptors by carbachol [5]. Subsequent work indicated that muscarinic receptor antagonist atropine could alleviate sAPPα release induced by ganstigmine and donepezil treatment [23]. Rivastigmine-induced sAPPα secretion increase can be blocked by scopolamine pretreatment suggest that rivastigmine might first bind with muscarinic receptor and then exert its pharmacological action on APP processing. That is, cholinergic transmission signaling pathway might be involved in rivastigmine induced sAPPα secretion, or, α-secretase activity regulation.

Secretion of sAPPα by α-secretase is a highly regulated second messenger pathway that involves several protein kinases including PKC, protein kinase A (PKA), MAPK, tyrosine kinase and phosphatidyl inositol 3 kinase (PI-3K) etc [21, 24–26]. The dada here that the increased sAPPα by rivastigmine can be blocked by PKC, ERK and JNK inhibitors indicate that rivastigmine may first activate these signal path-

ways and then exert its pharmacological effects. These findings are in line with previous data in relation to the involvement of these signaling pathways in regulating sAPPα release [24, 26, 27]. Since the actions of PKC, ERK and JNK are extremely complicated, a thorough analysis is needed to establish the mechanism of rivastigmine on sAPPα secretion.

It must be pointed out that the data here were obtained from in vitro study, and further work on the effects of rivastigmine on sAPPα and Aβ release in CSF and platelets from AD patients are need to be explored.

5. CONCLUSIONS

In conclusion, we show here that cholinesterase inhibitor rivastigmine can modulate APP processing by increasing sAPPα secretion and decreasing Aβ release in SK-N-SH neuroblastoma cells. Studies have shown that sAPPα can rescue neurons from neurotoxin insults such as Aβ, and drive expression of some survival genes such as transthyretin, insulin-like growth factor 2, insulin-like growth factor binding protein 2, etc. [28]. ChEIs are the mainstays of drugs approved in the USA and Europe and China for AD therapy. They are thought to increase acetylcholine levels in the brain via inhibition of acetylcholine degradation by acetylcholinesterase. We propose that in addition to their cholinergic effects, the neuroprotection offered by sAPPα and the decreased neurotoxic property of Aβ may also underlie the clinical effects observed in some AD patients after rivastigmine administration.

6. ACKNOWLEDGMENTS

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