Effects of galantamine on β-amyloid release and beta-site cleaving enzyme 1 expression in differentiated human neuroblastoma SH-SY5Y cells

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ABSTRACT

Galantamine (Gal) is an acetylcholinesterase inhibitor and used to treat the symptoms of Alzheimer’s disease (AD). Recent studies show that Gal may affect amyloid precursor protein (APP) metabolism and increase release of α-secretase APPs (sAPPα). However the effect of Gal on amyloid-β peptide (Aβ) release and β-site cleaving enzyme 1 (BACE1) expression is still unknown. Consequently, we investigated the effect of Gal on the level of Aβ and BACE1. In a differentiated human neuroblastoma cell line (SH-SY5Y), Gal (0.3 μM) was found to significantly decrease Aβ release and BACE1 expression following treatment for 6, 12, and 24 h. Increasing Gal to 0.9 μM or 10 μM had no further effect. The effect of Gal (0.3 μM for 18 h) was maximal on BACE1 expression but not on Aβ secretion. At higher concentration (0.9 μM and 10 μM), Gal had no effect on the level of full-length APP but could still stimulate further decrease in Aβ secretion and release of sAPPα. These observations suggested that 0.3 μM Gal exerts its effect on Aβ production by inhibiting BACE1 expression, while 0.9 μM or 10 μM Gal mainly reduces Aβ production by stimulating the non-amyloidogenic pathway to decrease the amount of APP substrate available for β-secretase cleavage. In addition, α7 nicotinic acetylcholine receptor (α7nAChR) and multiple second messengers (including PKC, MEK, and p38MAPK) were found to be involved in the regulation of Gal-inhibited Aβ release and BACE1 expression.

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1. Introduction

One of the hallmarks of Alzheimer’s disease (AD) is the presence in the brain of extracellular senile plaques composed mainly of extracellular deposits of amyloid-β peptide (Aβ) (Selkoe, 1994). Aβ is generated from the amyloid precursor protein (APP) through an initial cleavage with β-secretase at the amino-terminal side of the Aβ sequence to generate a soluble peptide, secretory APPs (sAPPs), and an intracellular carboxy-terminal fragment C99. Thereafter, γ-secretase cleaves C99 to release Aβ (Haass et al., 1992; Shoji et al., 1992). In the non-amyloidogenic pathway, α-secretase cleaves APP within the Aβ peptide sequence to generate a secreted form of APP fragment (sAPPα), preventing the formation of Aβ peptide (Esch et al., 1990; Selkoe et al., 1990), α- and β-secretases are believed to compete for APP, and thereby determine the amount of Aβ (Buxbaum et al., 1998). In one study, overexpression of α-secretases shifted APP processing towards the non-amyloidogenic pathway and resulted in decreased Aβ generation (Postina et al., 2004). The most likely candidate α-secretases are a membrane-bound disintegrin and metalloprotease (ADAM)10 and ADAM17 (Lammich et al., 1999; Nunan and Small, 2000). β-site APP cleaving enzyme 1 (BACE 1; an aspartic protease) was identified as a candidate β-secretase (Sinha et al., 1999; Vassar et al., 1999).

Galantamine (Gal), a plant-derived alkaloid, is a third-generation acetylcholinesterase inhibitor (AChEIs) (Maelicke et al., 2001). Gal treatment has been shown to alleviate cognitive deficits in patients with AD (Raskind et al., 2000; Tariot et al., 2000). In contrast to other AChEIs, such as tacrine, donepezil, and rivastigmine, Gal has a weak cholinesterase inhibitor effect. However, it interacts allosterically with nicotinic acetylcholine receptors (nAChRs) to potentiate the action of agonists of these receptors and enhance the sensitivity of the receptors to acetylcholine (Maelicke et al., 2001; Texido et al., 2005). Recent findings have demonstrated that Gal is also involved in APP processing (Lenzken et al., 2007).

The purpose of the present study was to determine the effect of Gal on Aβ generation and BACE1 expression in the differentiated human neuroblastoma cell line (SH-SY5Y), which expresses choline acetyltransferase and both muscarinic and nicotinic receptors (Adem et al., 1987; Gould et al., 1992; Halvorsen et al., 1995). Our findings may suggest an additional mechanism of the pharmacological action of Gal on APP metabolism.
dimethylsulfoxide and stored at −20 °C. Gal, atropine (a muscarinic acetylcholine receptor antagonist), and methyllycaconitine (MLA, an α7-nicotinic acetylcholine receptor antagonist) all from Sigma Chemical Co. were each dissolved in distilled water and stored at −20 °C. Trans-retinoic acid (Sigma Chemical Co.) was dissolved in dimethylsulfoxide and stored at −20 °C. All stocks were diluted in medium to their respective working concentrations immediately before use. Aβ1-40 colorimetric sandwich ELISA kit was obtained from Invitrogen (Carlsbad, CA, USA) and ELISA kit for Aβ1-42 was from Covance (Denver, PA). We also purchased the following antibodies: polyclonal APP antibody, which recognizes residues 44–60 of APP (Sigma), anti-BACE1 polyclonal antibody (Abcam, Cambridge, UK), anti-β-actin antibody (Santa Cruz Biotechnology), and anti-sAPPα (2B3) mouse IgG MAb (Immunobiological Laboratories Co., Ltd., Tokyo Japan). All culture media, supplements, and fetal bovine serum (FBS) were from Gibco (Carlsbad, CA, USA). Electrophoresis reagents were from Bio-Rad (Hercules, CA, USA). All other reagents were of the highest grade available and were purchased from Sigma Chemical Co. unless otherwise indicated.

2.2. Cell cultures

The human neuroblastoma SH-SY5Y cells (Institute of Basic Medical Sciences Chinese Academy of Medical Sciences) were cultured in medium with equal amount of Eagle's minimum essential medium and Nutrient Mixture Ham's F-12, supplemented with 10% FBS, glutamine (2 mM), penicillin/streptomycin, non-essential amino acids, at 37 °C in 5% CO2 and 95% air. For differentiation, cells were plated at a density of 5×104 cells/cm2 in 60-mm diameter culture dishes and exposed to 10 μM all-trans-retinoic acid for 6 days as previously described (Lenzken et al., 2007). Differentiation was considered to be complete when the growth of cone-terminated neurites was up to three times longer than one cell body diameter.

2.3. Treatment of cells with Gal and cell signaling inhibitors

Cells grown to 80% confluency were washed with serum-free medium, maintained in serum-free medium for 2 h, and then treated with Gal (0, 0.3, 0.9, or 10 μM/mL for 6, 12, 18, or 24 h). In inhibition studies, cells were exposed to 2.5 μM GF109203X, 30 μM PD98059, 30 μM SB203580, 10 μM LY294002, and 20 μM SP600125, which are inhibitors of protein kinase C (PKC), mitogen-activated protein kinase kinase (MEK), p38 mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase (PI3K), and c-Jun N-terminal protein kinase (JNK), respectively. Atropine (10 μM) and MLA (10 μM) were also used. Cells were exposed to inhibitors for 30 min before Gal treatment.

After incubation with the drugs for the indicated periods, conditioned media were collected and mixed with a complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). The media were centrifuged (13,000 × g for 5 min) to remove detached cells and debris, and supernatants were concentrated with centrifugal filter devices (Amicon Ultra-4; Millipore Corp., Bedford, MA) and then stored at −70 °C. Cell monolayers were washed twice with ice-cold PBS, lysed on the tissue culture dish by addition of ice-cold RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate, and protease inhibitors cocktail), and centrifuged (14,000 × g for 30 min at 4 °C). The supernatants were transferred to new microtubes and stored at −70 °C. Protein levels were determined using the BCA Protein Assay kit (Beyotime Institute of Biotechnology, Shanghai, China) and the amount of protein on each blot was equalized by loading a volume of sample of conditioned medium standardized to total cell lysate protein concentration.

2.4. Western blot assay

SDS-polyacrylamide gel electrophoresis (PAGE; 7.5% gel) was carried out on each sample (30 μg of protein). Separated proteins were subsequently transferred to 0.2-μm nitrocellulose membranes at 16 V for 30 min. The membranes were blocked with 5% fat-free milk in Tris-buffered saline containing 0.05% Tween 20 (TBST; 2 h at RT) and washed in TBST. The blots were probed with polyclonal antibodies to APP N-terminal (1:200 dilution) and BACE1 (1:200), and monoclonal antibodies to sAPPα (1:50) and β-actin (1:2000) at 4 °C overnight, washed for 30 min in TBST, incubated with horseradish peroxidase conjugated goat anti-mouse IgG and goat anti-rabbit IgG (both at 1:5000) for 2 h at RT, and visualized using a DAB kit. Densitometric quantification of blots was carried out using Alphapart11Ease version 5.0, and a ratio relative to control was calculated.

2.5. Quantification of secreted Aβ levels

Aβ released from control and drug-treated cells into the cell culture media was measured in lyophilized samples using ELISA kits for Aβ1-40 and Aβ1-42 according to the manufacturer’s protocol. The release of Aβ1-40 and Aβ1-42 was evaluated using standard curves generated in duplicate. The quantity of Aβ in each sample was measured in duplicate and expressed as mean ± standard error of the mean. Aβ1-40 and Aβ1-42 levels are expressed in pg/ml.

2.6. Statistical analysis

Data are presented as the mean ± the standard error of the mean (S.E.M). Each procedure was performed in duplicate in four independent experiments. Statistical analyses were carried out using one-way ANOVA, followed by a least significant difference post-hoc test. A difference at p < 0.05 was considered to be significant. All statistical analyses were performed using SPSS software version 13.0.

3. Results

3.1. Effect of Gal on Aβ1-40 and Aβ1-42 levels

Aβ1-40 and Aβ1-42 peptide levels were measured in medium conditioned by differentiated SH-SY5Y cells. Gal (0.3 μM) for 6, 12, and 24 h significantly decreased Aβ1-40 and Aβ1-42 generation. The magnitude of the effect was unaffected by further increases in Gal concentration (0.9 or 10 μM). However, the decrease in Aβ1-40 level (in pg/ml) after 18 h of Gal treatment was concentration-dependent: from 119.05 ± 2.01 to 95.25 ± 1.16 for 0.3 μM, 89.14 ± 2.96 for 0.9 μM, and 81.06 ± 2.28 for 10 μM Gal (Fig. 1A, p < 0.001). Also Aβ1-42 level decreased similarly: from 38.35 ± 0.48 to 32.10 ± 0.87 for 0.3 μM, 28.07 ± 1.05 for 0.9 μM, and 24.18 ± 0.24 for 10 μM Gal (Fig. 1B, p < 0.001).

3.2. Effect of Gal on BACE1 and cellular APP expression

BACE1, which plays an essential role in Aβ generation, was monitored. Gal (0.3 μM) for 6, 12, 18, and 24 h significantly decreased the expression of BACE1. However, further increase in the concentration of Gal increased BACE1 expression. Gal was maximally effective at a concentration of 0.3 μM after 18 h of treatment, which resulted in a 44% decrease in BACE1 level compared with the control level (Fig. 2, p < 0.001). Western blot analysis of the effect of Gal (after treatment for 6, 12, 18, and 24 h) on cellular APP levels found no effect on APP steady-state levels (Fig. 3).

3.3. Effect of Gal stimulation on sAPPα release

Western blotting was used to determine the amount of sAPPα released in serum-free medium by differentiated SH-SY5Y cells exposed to Gal (0–10 μM) for 18 h. The effect of Gal on the release of sAPPα proved to be concentration-dependent, with the maximal effect (24.5-fold increase above control level) observed at a concentration of 10 μM Gal (Fig. 4, p < 0.001)
3.4. Pharmacological characterization of the cholinergic effect of Gal

To determine whether Gal decreases Aβ or BACE1 level through receptor mediated mechanisms, differentiated SH-SY5Y cells were exposed to 0.3 μM Gal in the presence or absence of 10 nM atropine or 10 nM MLA. Treatment with these inhibitors alone had no effect on BACE1 expression (Fig. 5, Table 1) or Aβ1-40 and Aβ1-42 secretion (Table 1). Treatment (18 h, 0.3 μM) with Gal reduced the level (in pg/ml) of Aβ1-40 from 119.56 ± 2.22 to 97.00 ± 3.25 (p < 0.001) and of Aβ1-42 from 38.49 ± 1.46 to 32.38 ± 0.72 (p < 0.001). Addition of MLA reversed this effect, restoring the Aβ1-40 level to 116.74 ± 3.98 pg/ml (p > 0.05) and the Aβ1-42 level to 37.24 ± 1.34 pg/ml (p > 0.001). Pre-treatment with 10 nM atropine had no effect on the Gal-induced reduction in Aβ1-40 or Aβ1-42 level, which was 95.93 ± 3.15 pg/ml and 32.10 ± 0.64 pg/ml, respectively (Table 1).

Similarly, 10 nM MLA inhibited the Gal-induced decrease in BACE1 expression by 82.7% (p < 0.001), while 10 nM atropine had no effect on this decrease (Fig. 5, Table 1).

3.5. Signal transduction molecules involved in Gal-induced decrease in BACE1 expression and Aβ release

To identify the signal transduction pathways involved in this effect, several protein kinase inhibitors were added prior to Gal (0.3 μM) treatment. Treatment with these inhibitors alone had no effect on BACE1 expression (Fig. 5, Table 1) or Aβ1-40 and Aβ1-42 secretion (Table 1), compared to control levels. Pre-treatment with PKC inhibitor GF109203X (2.5 μM), MEK inhibitor PD98059 (30 μM), p38MAPK inhibitor SB203580 (30 μM), however, prevented Gal-induced decrease in Aβ1-40 and Aβ1-42 secretion, raising Aβ1-40 level (in pg/ml) to 102.30 ± 3.37 (p < 0.05), 113.92 ± 1.03 (p < 0.001), and 113.54 ± 1.14 (p < 0.001), respectively and Aβ1-42 level to 34.74 ± 0.87 (p < 0.05), 36.68 ± 0.48 (p < 0.001), and 36.40 ± 1.27 (p < 0.001), respectively. Pre-treatment with PI3-K specific inhibitor LY294002 (10 μM) and the JNK pathway inhibitor SP 600125 (20 μM), on the other hand, failed to prevent either the Gal-induced decrease in level of Aβ1-40 (which was 96.23 ± 2.74 pg/ml in the presence of LY + Gal and 96.50 ± 1.35 pg/ml in the presence of SP + Gal) or Aβ1-42 (which was 33.90 ± 1.58 pg/ml in the presence of LY + Gal and 27.24 ± 0.24 pg/ml in the presence of SP + Gal) (Table 1).

These inhibitors had similar effects on Gal-induced changes in BACE1 expression. The effect of Gal on BACE1 was reduced approximately 54.1%, 59.7%, and 34.2%, respectively, by pre-treatment with GF109203X (2.5 μM), PD98059 (30 μM), and SB203580 (30 μM), compared to Gal-treated cultures without inhibitors (p < 0.001). However, LY294002 and SP600125 failed to modulate Gal-induced changes (Fig. 5, Table 1).

4. Discussion

AChEIs are mainly used for the treatment of AD (Giacobini, 2000). The clinical efficacy has been related to the ability of the drug to inhibit acetylcholinesterase activity, thus preventing the hydrolysis of...
APP in the cell lysates were detected by APP polyclonal antibody. (A) Representative Western cells. Cells were incubated with Gal (0, 0.3, 0.9, or 10 \( \mu \)M) for 6, 12, 18, and 24 h. Full-length APP in the cell lysates were detected by APP polyclonal antibody. (A) Representative Western blot showing APP bands (~125 kDa). (B) Densitometric analysis of APP protein levels normalized to \( \beta \)-actin level. Results are shown as the mean ± SEM for each condition and tested for statistical significance using repeated-measures one-way ANOVA with post-hoc LSD test (n = 4 for each condition).

**Fig. 3.** Galantamine had no effect on the steady-state levels of APP in differentiated SH-SY5Y cells. Cells were incubated with Gal (0, 0.3, 0.9, or 10 \( \mu \)M) for 6, 12, 18, and 24 h. Full-length APP in the cell lysates were detected by APP polyclonal antibody. (A) Representative Western blot showing APP bands (~125 kDa). (B) Densitometric analysis of APP protein levels normalized to \( \beta \)-actin level. Results are shown as the mean ± SEM for each condition and tested for statistical significance using repeated-measures one-way ANOVA with post-hoc LSD test (n = 4 for each condition).

Released acetylcholine, increasing the efficiency of cholinergic transmission, and reducing memory and cognitive impairment. In addition to improving disease symptoms, AChEIs may impact biochemical pathways involved in APP processing. Lenzken et al. (2007) demonstrated that Gal (10 \( \mu \)M) for 2 h promoted a strong increase (on average three times baseline value) in sAPP\textalpha release. Peng et al. (2007) showed that the acetylcholinesterase inhibitor, huperzine A (Hup A), also increased sAPP\textalpha secretion and that this increase involved ADAM10 and tumor necrosis factor (TNF)-alpha convertase (TACE)/ADAM17. However, the effect of Gal on \( \alpha \text{-}7 \) level and BACE1 expression is still unknown.

Our study demonstrated a Gal-induced decrease in \( \alpha \text{-}7 \) levels in differentiated SH-SYSY cells, but the precise mechanism is still unclear. Three mechanisms are possible: (i) decreased synthesis of APP after Gal treatment; (ii) Gal stimulation of the non-amyloidogenic pathway resulting in decrease of the APP substrate available for \( \beta \)-secretase cleavage; (iii) Gal-induced decrease in \( \beta \)-secretase activity. Consequently, we investigated the effect of Gal on the expression of APP, BACE1, and sAPP\textalpha.

In agreement with a previous study (Lenzken et al., 2007), we found that Gal had no effect on the steady-state levels of APP, indicating that Gal-inhibited secretion of \( \alpha \text{-}7 \) was due to the reduced cleavage of APP via the \( \beta \)-secretase pathway rather than decreased synthesis of APP. We also observed a concentration-dependent effect of Gal (in the range, 0–10 \( \mu \)M) on both BACE1 expression and \( \alpha \text{-}7 \) secretion after 18 h of exposure. The effect of Gal on BACE1 expression was maximal at 0.3 \( \mu \)M, but \( \alpha \text{-}7 \) secretion was decreased further at higher Gal concentration, which suggested that at these higher concentrations (0.5 and 10 \( \mu \)M) Gal was acting on a mechanism other than decreasing \( \beta \)-secretase activity. Therefore, higher concentrations (0–10 \( \mu \)M) of Gal were used in subsequent experiments on sAPP\textalpha level.

Interestingly, treatment with Gal (0.9 and 10 \( \mu \)M) for 18 h was found to stimulate sAPP\textalpha release, which suggests that Gal in higher concentration can shift APP processing towards the non-amyloidogenic pathway and thereby possibly decrease \( \alpha \text{-}7 \) generation. Overall, it appears that the effect of Gal on \( \alpha \text{-}7 \) production is complex and concentration-dependent. We speculate that Gal at lower concentration affects \( \alpha \text{-}7 \) production by inhibiting BACE1, while at higher concentrations it mainly reduces \( \alpha \text{-}7 \) production via the non-amyloidogenic pathway and thereby reduces the availability of APP substrate for \( \beta \)-secretase cleavage.

It is worth mentioning that the effective concentration of galantamine for significantly decreasing levels of BACE1 and \( \alpha \text{-}7 \) in our experiment (0.3 \( \mu \)M) differs from its ICSO (1.85–16 \( \mu \)M) for blocking AChE (Arias et al., 2004; Lenzken et al., 2007; Thomsen et al., 1991). It therefore seems that the galantamine activities reported here are not directly related to AChE inhibition and may be mediated in a receptor-dependent manner. Several studies have demonstrated the involvement of the cholinergic receptor in APP processing. However, the main focus has been on the role of the cholinergic receptor in the non-amyloidogenic pathway of APP processing (Lenzken et al., 2007; Peng et al., 2007; Zimmermann et al., 2004). Only a few studies have investigated the involvement of \( \alpha \text{-}7 \) nAChRs in the regulation of \( \alpha \text{-}7 \) release or BACE1 expression. Srivareerat et al. (2009) indicated that 6 weeks of nicotine treatment reduced the levels of Abeta (1–40) and BACE1 peptides in the hippocampal area CA1 and prevented Abeta-induced impairment of learning and short-term memory in AD rats. Hellstroem-Lindahl et al. (2004) also found that long- and short-term nicotine treatment significantly reduces the amount of insoluble \( \alpha \text{-}7 \) and \( \alpha \text{-}7 \)–42 in brains from APP\textalpha mice. This reduction might be, in part, mediated via the \( \alpha \text{-}7 \) nicotinic receptor. However, nicotine has a negative effect on both \( \alpha \text{-}7 \) and \( \beta \)-secretase activities. Since data from Lenzken et al. (2007) show that Gal modulates non-amyloidogenic processing of APP in a fully receptor-dependent manner (mainly through \( \alpha \text{-}7 \)nAChR), we herein investigated whether the action of Gal on amyloidogenic processing of APP involved the participation of
We found that 10 nM MLA largely prevented both Gal-inhibited Aβ1-40 release and BACE1 expression, suggesting that modulation of BACE1 expression and Aβ1-40 release involved α7nAChR. These findings further support our proposal that Gal-induced modulation of the amyloidogenic processing of APP involves α7nAChR rather than cholinergic enhancement due to AChε inhibition.

Several publications argued that atropine (10 μM) may also affect nicotinic receptors and thereby lead to overestimates of the importance of muscarinic receptors (Efthimiopoulos et al., 1996; Racchi et al., 2001; Mazzucchelli et al., 2003). Therefore, we used 10 nM atropine, a concentration putatively blocking only muscarinic receptors. We observed no blocking effect of atropine on either Gal-modulated BACE1 expression or Aβ1-40 release. Considering the evidence provided by Lenzken et al. (2007) that 10 nM atropine extensively blocks (60%) the effect of carbachol (100 μM), a non-specific cholinergic receptor agonist, and not that of Gal, we hypothesized that muscarinic receptors were not involved in the action of Gal described here.

To investigate the role of various second messengers in the Gal-modulated Aβ1-40 release and BACE1 expression, cells were treated with inhibitors shown to be involved in APP processing (i.e., PKC, MAPK, PI3K inhibitors) before treatment with Gal (Mazzucchelli et al., 2003; Solano et al., 2000; Yogev-Falch et al., 2002). PKC inhibitors and various MAP kinase inhibitors partially reversed the effect of Gal on Aβ1-40 release, suggesting the involvement of the PKC pathway and p38 MAPK and MEK in Gal-modulated Aβ1-40 release. However, the failure of LY294002 or SP600125 pre-treatment to block the effect of Gal seemed to exclude a role for the PI3K and JNK pathways. The effect of inhibitor pre-treatment on BACE1 expression was similar, which supported our proposal that Gal (0.3 μM) reduces Aβ production through BACE1 reduction. However, details of the activation of these signaling molecules and their relationship to α7nAChR deserves further investigation.

In conclusion, our data indicate that Gal inhibited both Aβ generation and BACE1 expression and that this effect involves α7nAChR and several signal transduction molecules such as PKC, p38MAPK, and MEK.

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