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Amylin Enhances Amyloid- β Peptide Brain to Blood Efflux Across the Blood-Brain Barrier

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Abstract

Findings from Alzheimer's disease (AD) mouse models showed that amylin treatment improved AD pathology and enhanced amyloid- β (A β) brain to blood clearance; however, the mechanism was not investigated. Using the Tg2576 AD mouse model, a single intraperitoneal injection of amylin significantly increased A β serum levels, and the effect was abolished by AC253, an amylin receptor antagonist, suggesting that amylin effect could be mediated by its receptor. Subsequent mechanistic studies showed amylin enhanced A β transport across a cell-based model of the blood-brain barrier (BBB), an effect that was abolished when the amylin receptor was inhibited by two amylin antagonists and by siRNA knockdown of amylin receptor Ramp3. To explain this finding, amylin effect on A β transport proteins expressed at the BBB was evaluated. Findings indicated that cells treated with amylin induced LRP1 expression, a major receptor involved in brain A β efflux, in plasma membrane fraction, suggesting intracellular translocation of LRP1 from the cytoplasmic pool. Increased LRP1 in membrane fraction could explain, at least in part, the enhanced uptake and transport of A β across the BBB. Collectively, our findings indicated that amylin induced A β brain to blood clearance through amylin receptor by inducing LRP1 subcellular translocation to the plasma membrane of the BBB endothelium.

Keywords

Alzheimer's disease; amylin; amyloid- β ; blood-brain barrier; LRP1

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia. While the number of AD patients grows rapidly in the US [1, 2], there are no effective treatments for the disease. Major hallmarks of AD include amyloid- β (A β) deposits in brain parenchyma, also known

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as senile plaques, neurofibrillary tangles of hyper-phosphorylated tau protein, and neuronal loss [3, 4]. Failure in A β clearance from the brain is considered a major factor that contributes to A β brain accumulation in sporadic or late onset AD [5–7]. Clearance of A β from the brain is mediated through three major pathways: first, efflux across the blood-brain barrier (BBB) [8, 9], second, degradation by proteolytic enzymes such as insulin degrading enzyme (IDE) [10] and neprilysin [11, 12], and third, by bulk flow of interstitial fluid [13, 14]. Clearance of A β across the BBB is mediated by several transport proteins mainly low-density lipoprotein receptor-related protein-1 (LRP1) expressed on the brain side of the BBB (abluminal); the protein mediates efflux of A β from the brain into the peripheral circulation across the BBB [9, 15, 16]. In AD and in aged rats, the expression of LRP1 at the BBB is markedly decreased, which contributes to reduction in A β brain clearance and its increased accumulation inside the brain [6, 17]. Therefore, enhancing A β clearance across the BBB by inducing its transport-based clearance mechanism has been considered as a potential therapeutic approach to reduce A β brain burden [17–20]. However, there is no effective such drug yet for AD.

Amylin is a 37 amino acid peptide hormone that is co-secreted with insulin by beta cells of the pancreas, the most well-known site for amylin production [21]. Through binding to its receptor, which is composed of the calcitonin receptor (CTR) complexed with different receptor-activity-modifying protein (RAMP) [22], amylin plays a pivotal role in glycemic regulation by controlling several biological processes including: slow gastric emptying, induce satiation, and prevention of postprandial increase in plasma glucose level [23, 24]. Amylin crosses the BBB and enters the brain where it is suggested to have a role in glucose metabolism, neural regeneration, and have cerebral vasculature effects [25, 26]. Furthermore, amylin is important in regulating cellular metabolism and survival by interacting with insulin signaling pathways and downstream pathways like AMP-activated protein kinase [27]. Amylin and A β share several features, including having similar β -sheet secondary structures [28], binding to the same amylin receptor [29], and being degraded by the same protease, insulin degrading enzyme (IDE) [10]. Thus, the accumulation of A β as well as amylin aggregation in AD brains may interrupt monomeric amylin's essential brain functions by blocking amylin receptors [24, 30].

Two recent studies, including our own, reported the beneficial effect of chronic peripheral injection of amylin or its analog pramlintide on brain A β pathology and cognitive impairment in murine models of AD [31]. Findings showed reduced behavioral impairment and brain A β deposition, which was associated with increased A β plasma levels; however, the mechanism by which amylin induced such effect was not clear. Herein, we sought to study the possible role of amylin in regulating the clearance of A β from the brain to the blood across the BBB. For this, we investigated the effect of a single intraperitoneal injection of amylin, with or without the preinjection of amylin receptor inhibitor on A β serum levels in an AD mouse model. Then, we elucidated the mechanism by which amylin influence A β transport across the BBB using an *in vitro* BBB model.

MATERIALS AND METHODS

Materials

Amylin (human, Cat# AS-60254-1) and AC253 were purchased from AnaSpec (Fremont, CA). Acetyl-[Asn30, Tyr32] sCT(8–37) (AC187) was purchased from Tocris Bioscience (Minneapolis, MN). Synthetic monoiodinated and nonoxidized A β ₄₀ (¹²⁵I-A β ₄₀; human, 2200 Ci/mmol) was purchased from PerkinElmer (Boston, MA). Fetal bovine serum (FBS) was purchased from ATLANTA biological (Flowery Branch, GA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO). Total protein measurement's reagents with the bicinchoninic acid (BCA) method were obtained from Pierce (Rockford, IL). For western blot, mouse monoclonal antibody against light chain LRP1 was obtained from Abcam (Cambridge, MA), CAT# ab92544; goat polyclonal antibodies against actin (C-11), CAT# sc-1615, and HRP-labeled secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

Mice and experimental treatments

Tg2576 (APP^{sw}, K670N/M671L) transgenic mice [32] were originally purchased from Taconic (Hudson, NY) and were maintained on a B6SJLF1/J background at the Boston VA animal facility before this study. All mice were maintained in micro isolator cages in the Animals Facility of Boston University School of Medicine. Female Tg2576 mice aged 9 months were used. Before the challenge experiments with amylin, the mice received a baseline blood draw, and then were treated either with intraperitoneal injection (i.p.) of AC253 (200 μ g/kg), the amylin receptor antagonist, or of phosphate-buffered saline (PBS) on daily basis for 3 days ($n = 4$ for each group). 24 h after the third injection, blood draw was conducted for each mouse followed by a single i.p. injection of amylin (200 μ g/kg) as a challenge. 24 h after the challenge, the final blood draw was conducted. All blood samples were collected to isolate serum. All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Boston University Animal Care and Use Committee. The experiments have been reported in compliance with the ARRIVE guidelines.

Determination of serum A β levels

The A β levels in serum were assayed by an ELISA method [33, 34] in which A β was trapped with either monoclonal antibody to the C-terminal of A β ₄₀ (2G3) or A β ₄₂ (21F12) (from Dr. Dennise Selkoe) and then detected with biotin-conjugated to the N-terminus of A β . A dilution of 6E10 was optimized to detect A β in the range of 50 to 800 pg protein in the brain samples. The dilution of blood was between two- and five-fold; the lowest detection level for each A β peptide in blood was 1.6 pg/ml determined from a standard curve. Streptavidin-conjugated alkaline phosphatase (Promega; Madison, WI) was added and incubated, and the signal was amplified by adding alkaline phosphatase fluorescent substrate (Promega), which was then measured by a plate reader (BioTek; Winooski, VT).

Cell culture

The mouse brain endothelial cells (bEnd3; ATCC, Manassas, VA), passage 27–33, were cultured in DMEM growth medium supplemented with 10% FBS, and the antibiotics penicillin G (100 units/mL) and streptomycin (100 µg/mL). The cells were grown to confluence in 75 cm² cell culture flasks for one to two days in a humidified atmosphere (5% CO₂/95% air) at 37°C. Monomeric amylin (confirmed by western blot) was prepared according to the manufacturer instructions in water and immediately aliquoted and stored at –20°C. To prepare the required concentrations, amylin aliquots were diluted in cell culture media.

Uptake of ¹²⁵I-Aβ₄₀ by bEnd3 cells after treatment with amylin

For the uptake study, cells were cultured on 24-well plate and grown to confluence. Cells were treated with 5 or 10 µM of amylin or media for 45 min, followed by addition of media containing 0.1 nM of ¹²⁵I-Aβ₄₀ and 5 or 10 µM amylin for 15 min. For amylin receptor inhibition studies, cells were pre-incubated for 45 min with 5 µM amylin with or without AC187 (5 µM) and AC253 (10 µM), or with media followed by addition of media containing 0.1 nM of ¹²⁵I-Aβ₄₀ with corresponding treatment and then incubated for 15 min. At the end of the experiment, medium was aspirated and cells were washed three times with ice-cold PBS while keeping plates on ice. Cells were then lysed by the addition of RIPA buffer containing 1% protease inhibitors cocktail, by gentle shaking on a rocking platform for 2 h at 4°C. Intracellular radioactivity of ¹²⁵I-Aβ₄₀ was measured using a Wallac 1470 Wizard Gamma Counter (PerkinElmer Inc. Waltham, MA). Since bEnd3 cells only express Ramp3, to further confirm the enhanced Aβ uptake caused by amylin treatment is mediated by amylin receptors, siRNA studies to knock down the amylin receptor component Ramp3 were performed. Ramp3 Silencer[®] select siRNA (Ambion, NY) was used to silence Ramp3 receptor. Briefly, 9000 cells/cm² were seeded in 6-well plates. At 70–90% confluency, cells were transfected with 100 nM Ramp3 siRNA using lipofectamine 2000[®] (Invitrogen, CA) following the manufacturer protocol. Four hours after transfection, cells were washed and lysed to quantify knockdown efficiency. Quantitative real-time PCR and gel analysis demonstrated 84–90% reduction in Ramp3 gene expression. For ¹²⁵I-Aβ₄₀ uptake studies, 4 h after transfection cells were directly washed and used for amylin treatment and Aβ uptake as described above.

Uptake studies of ¹²⁵I-Aβ₄₀ with amylin analog, pramlintide, were also performed for comparison at the concentrations 0.1, 0.25 and 1.0 µM.

Transport of ¹²⁵I-Aβ₄₀ across the BBB model after treatment with amylin

bEnd3 cells cultured on inserts were treated for 45 min with 0.0, 0.05, 0.25, 1, and 5 µM of amylin prepared in media. The aforementioned treatments were applied to the apical side (A, upper chamber representative of the blood side) in one set of experiments and to the basolateral side (B, lower chamber representative of the brain side) in another set of experiments. The basolateral to apical transport (B→A) of Aβ was initiated by addition of media containing 0.1 nM of ¹²⁵I-Aβ₄₀ and 0.1 µCi/ml of ¹⁴C-inulin, a paracellular permeability marker, to B side for 30 min.

For amylin receptor inhibition studies, cells were pre-incubated with increasing concentrations of AC187 at 0.0, 0.05, 0.25, 1, 5 μM together with 1 μM amylin for 45 min added to the apical side. A β transport was initiated by adding 0.1 nM of ^{125}I -A β_{40} and ^{14}C -inulin to the basolateral side and incubated for 30 min. Aliquots from both A and B sides were sampled for radioactivity analysis using Wallac 1470 Wizard Gamma and Wallac 1414 WinSpectral Liquid Scintillation Counters (PerkinElmer). Degradation of ^{125}I -A β_{40} was determined by the trichloroacetic acid (TCA) precipitation assay [35]. Transport quotient (TQ) of ^{125}I -A β_{40} from B $\rightarrow$ A was calculated using the following equation [36].

$$A\beta_{40}TQ = \frac{(A\beta_{40}(\text{apical})) / (A\beta_{40}(\text{total}))}{(\text{Inulin}(\text{apical})) / (\text{Inulin}(\text{total}))} \quad (1)$$

where A $\beta_{40}(\text{total})$ is the sum of cpm of intact ^{125}I -A β_{40} in the apical and basolateral compartments, and inulin $_{(\text{total})}$ is the sum of dpm in the apical and basolateral compartments.

In addition and for comparison, transport studies of ^{125}I -A β_{40} with pramlintide were performed at the concentrations 0.01, 0.05 and 0.25 μM .

Determination of amylin transport across bEnd3 cell monolayer

To determine whether amylin is able to cross the BBB *in vitro* model, bEnd3 cells cultured on inserts were treated with 0.0, 0.05, 0.25, 1.0, or 5.0 μM of amylin prepared in media. Amylin was applied to the apical side in one set of experiments to initiate A $\rightarrow$ B transport, and to the basolateral side in another set of experiments to initiate B $\rightarrow$ A transport. After 45 min of incubation at 37°C, transport was stopped by placing plates on ice and samples were taken from both apical and basolateral sides for amylin analysis. Concentration-dependent transport of amylin from apical to basolateral (A $\rightarrow$ B) and basolateral to apical (B $\rightarrow$ A) were determined. Also, the ratio of amylin transport B $\rightarrow$ A/A $\rightarrow$ B was calculated.

Determination of amylin by ELISA

Samples were taken directly from media, centrifuged, and clear supernatant was diluted as required. Human amylin used in our experiments was measured using a monoclonal antibody-based sandwich immunoassay obtained from Millipore Corporation (Billerica, MA). Plates provided were coated with capture antibody, mouse anti-human amylin antibody, and for detection, antibody was anti-human amylin-alkaline phosphatase conjugate. The fluorescent signal was monitored at 355 nm/460 nm using Synergy 2 microplate reader supplied with Gene5 software for quantification (BioTek).

Western blot analysis

To study the effect of amylin on LRP1 protein expression, cells were seeded in 10 mm cell culture dishes (Corning, NY) at a density of 1×10^6 cells per dish and allowed to grow in a humidified incubator (5% CO₂/95% air) at 37°C. At 80–90% confluence, cells were treated with media or 1 μM of amylin for 1.5 h, or were pre-treated with 5 μM of AC187 for 15 min followed by co-treatment with 1 μM of amylin for 1.5 h. At the end of treatment period, cells

were washed with ice-cold PBS twice, scraped, collected, and lysed in RIPA buffer containing 1% protease inhibitors cocktail. Total protein was determined using the BCA protein assay. To obtain plasma membrane fraction we used Mem-PER Plus kit (Thermo Fisher Scientific; Waltham, MA) following the manufacturer instructions. This kit effectively solubilizes and isolates integral membrane proteins from cytosolic proteins using two different detergents: first with mild detergent to permeabilize the cells to release soluble cytosolic proteins, followed by a second detergent to solubilize membrane proteins. Samples were stored at -80°C until time of analysis. 25 μg of protein samples were loaded and resolved using 7.5% SDS-polyacrylamide gel at 140 V for 1 h and transferred electrophoretically onto nitrocellulose membranes at 300 mA for 3 h. After that, membranes were incubated in 2% BSA blocking solution with rocking for 1 h at room temperature followed by overnight incubation at 4°C with the primary antibodies for LRP1 (light chain) or β -actin (C-11). Proteins' blots were developed using a chemiluminescence detection kit (SuperSignal West Femto substrate; Thermo Fisher Scientific). Bands were visualized using C-DiGit® Blot Scanner (LI-COR Biosciences; Lincoln, NE) and band intensities were measured by densitometric analysis.

Statistical analysis

All data were expressed as mean $\pm$ S.E.M. The outcomes in mouse experiments for the effect of amylin challenge on $\text{A}\beta$ serum levels were statistically analyzed using one way ANOVA followed with posthoc analysis using a Dunnett's test. For the *in vitro* studies, one way ANOVA followed with posthoc analysis using a Dunnett's test, or Student's *t*-test was used to compare between samples. A *p*-value less than 0.05 were considered statistically significant. All statistical analyses were done using GraphPad Prism, version 5.03.

RESULTS

Effect of amylin on $\text{A}\beta$ serum levels

Since peripheral amylin treatment reduced the amyloid pathology in the brain but increased the concentrations of $\text{A}\beta_{42}$ in serum [31], we first tested whether amylin class peptides stimulate the removal of the toxic $\text{A}\beta$ from the AD brain to blood through amylin receptor. We measured $\text{A}\beta$ levels in serum before and after i.p injection of a single dose of amylin into Tg2576 mice, an AD mouse model, with and without pre-treatment of an amylin antagonist, AC253. None of the treatments caused adverse events after the i.p injections and all animals were used in the analysis. Generally, amylin treatment is safe and the major adverse event of amylin type peptide is hypoglycemia, however in our study design we did not see glucose change after one injection of amylin with glucose concentrations of 154.88 ± 4.14 mg/dl at baseline (i.e., before injections) and 155.76 ± 6.57 mg/dl after amylin treatment. A single dose of amylin injection in the AD mice produced a significant increase in serum $\text{A}\beta_{40}$ ($F = 23.87$; $p < 0.001$) and $\text{A}\beta_{42}$ ($F = 13.57$; $p < 0.001$) 24 h post the injection (Fig. 1). In contrast to pre-treatment with PBS, pre-treatment of these mice with AC253 abolished the amylin effect on the $\text{A}\beta$ surge in the blood, suggesting that amylin receptors may have role in enhancing $\text{A}\beta$ brain to blood clearance.

Amylin transport across the BBB in vitro model

Next, we evaluated amylin transport across the cell-based BBB model which mimics *in vivo* endothelium; bEnd3 cells were grown on filters to form a polarized monolayer for transport studies. As shown in Fig. 2A and B, the transport of amylin added to either side is concentration dependent and in both directions, A to B ($F = 78.17$; $p < 0.001$) and B to A ($F = 45.99$; $p < 0.01$) (A, apical or blood side; B, basolateral or brain side). While small percentage of the added amylin crosses the BBB model (less than 0.01% depending on the concentration), it appeared that amylin has better transport ability from A→B as compared to B→A transport as determined by calculating the ratio of A→B/B→A transport (shown in Fig. 2C; $F = 51.19$; $p < 0.01$) which was higher by up to 2.5-fold at 1 μM concentration of amylin. This result indicates that amylin could cross the BBB and move from blood (A) to brain side (B) and less vice versa suggesting amylin is preferentially transported from the general circulation to the brain. Moreover, these findings suggest amylin transport is not passive and is saturable process where its transport was not in direct proportion with the concentration (Fig. 2C). The addition of amylin did not show any effect on the monolayer integrity as measured by inulin paracellular transport (ranging from 0.33–0.44% with different concentrations of amylin compared to 0.41% with control; $p > 0.5$) confirming that the BBB model was not compromised by amylin treatment.

Effect of amylin on the uptake of ^{125}I -A β_{40} in bEnd3 cells

We then studied amylin effect on the uptake of exogenous A β by bEnd3 cells. As shown in Fig. 3A, incubation of cells with 5 and 10 μM of amylin significantly enhanced the uptake of ^{125}I -A β_{40} by approximately 3- and 4-fold, respectively ($n = 5$ per treatment, F -value = 52.99; $p < 0.001$). To test whether amylin effect on ^{125}I -A β_{40} uptake was mediated by amylin receptors, *in vitro* studies were performed first to confirm the *in vivo* findings with AC253, and second to compare the inhibitory effect of AC253 with another established amylin receptor antagonist, AC187, on reversing amylin effect. Pre-incubation of bEnd3 cells with 10 μM of AC253 significantly inhibited the effect of amylin on ^{125}I -A β_{40} uptake, by 13% (Fig. 3B, $n = 6$, F -value = 12.1; $p < 0.05$) as compared to amylin treatment alone. In addition, cells pre-treatment with 5 μM AC187 completely inhibited the effect of amylin on A β cellular internalization (Fig. 3B; $n = 6$, F -value = 12.1; $p < 0.05$). Treatment with AC253 or AC187 alone had no effect on ^{125}I -A β_{40} uptake confirming that the observed effect was caused by amylin through amylin receptor(s) (Fig. 3B). This result is consistent with reported high antagonistic potency of AC187 [37], and was thus used in subsequent studies. Interestingly, addition of amylin to the cells did not alter ^{125}I -A β_{40} cellular degradation as determined by the TCA assay; A β_{40} degradation measured in the medium ranged from 70–73% in all amylin and control treated cells (Fig. 3C; $n = 6$, $p > 0.05$).

We further used RNA silencing method to confirm the involvement of amylin receptor(s) in A β internalization by the endothelial cells. We and others have reported Ramp1 and Ramp3 are expressed in the mouse brain [38, 39]. Here, to confirm their expression in bEnd3 we first performed pilot studies to identify the amylin receptor expressed in these cells. Findings from PCR studies confirmed bEnd3 cells express Ramp3, a component of the amylin receptor ($n = 3$, t -value = 5.65; $p < 0.001$, Fig. 4A). In addition and as shown in Fig. 4B, knocking-down the expression of Ramp3 with Ramp3 siRNA in bEnd3 cells significantly

abolished the effect of amylin on ^{125}I -A β_{40} cellular uptake ($n = 3$, F-value = 15.84; $p < 0.01$). Consistent with amylin receptor inhibition studies with amylin antagonists, the data further confirmed role of amylin receptor in mediating increased cellular uptake of A β triggered by amylin treatment. These results suggest amylin enhanced A β uptake in endothelial cells through binding to the amylin receptor complexed with Ramp3.

Effect of amylin on transport of ^{125}I -A β_{40} across the BBB in vitro model

While amylin did not affect the integrity of the cell-based BBB model and increased A β uptake by the BBB cells (Figs. 2 and 3), we next examined if amylin treatment could increase A β transport across the BBB. As shown in Fig. 5, compared to control, B $\rightarrow$ A transport of ^{125}I -A β_{40} , measured as $\text{TQ}_{\text{B}\rightarrow\text{A}}$, was enhanced by 20 to 50% in the presence of different concentrations of amylin (0.05 to 5 μM) added to either side, to apical (Fig. 5A; $n = 4$, F-value = 5.401; $p < 0.05$) or basolateral (Fig. 5B; $n = 4$, F-value = 5.34; $p < 0.05$ for amylin 0.25 μM). On the other hand, addition of amylin to apical or basolateral side did not alter the degradation of ^{125}I -A β_{40} as determined by TCA assay where A β degradation in the media with or without amylin ranged between 69–71% in the apical side ($p > 0.5$), and in the range of 11–13% in the basolateral side ($p > 0.5$), which is consistent with our previous findings [36]. To further confirm the role of amylin receptor in mediating effect of amylin on ^{125}I -A β_{40} B to A transport, an amylin receptor antagonist was used. Addition of 0.05–5 μM of AC187 with amylin to the apical side caused a significant concentration-dependent reduction in amylin-induced ^{125}I -A β_{40} B $\rightarrow$ A transport (Fig. 5C; $n = 4$, F-value = 6.91; $p < 0.05$). This data indicated that amylin receptor is involved in amylin-induced transport of ^{125}I -A β_{40} across the *in vitro* BBB model.

Effect of pramlintide on uptake and transport of ^{125}I -A β_{40}

Pramlintide is an analog of amylin with three amino acid changes and an FDA approved drug for diabetes with a favorable safety profile [40]. Similar to amylin, we found that pramlintide enhanced ^{125}I -A β_{40} uptake by bEnd3 cells as well its transport across the *in vitro* BBB model. As shown in Fig. 6A, cells treatment with 0.1 and 0.25 μM of pramlintide significantly enhanced the uptake of ^{125}I -A β_{40} by 1.5–2.1-fold, respectively ($n = 6$, F-value = 98.7; $p < 0.001$). Interestingly pramlintide effect on augmenting ^{125}I -A β_{40} cellular uptake was lower at 1.0 μM (1.2-fold) than that at 0.25 μM but is significant when compared to the control ($p < 0.05$). While the explanation for this observation is not clear, this reduction at 1 μM concentration could be attributed to pramlintide aggregation at this concentration under the *in vitro* conditions used, induction of other mechanism that negatively impacted A β transport, or that pramlintide shares A β transport mechanism(s) that perturbed its uptake. According to these results A β transport studies were performed in the range of 0.01–0.25 μM pramlintide.

Similarly, as shown in Fig. 6B, B $\rightarrow$ A transport of ^{125}I -A β_{40} , measured as $\text{TQ}_{\text{B}\rightarrow\text{A}}$, was significantly enhanced in the presence of different concentrations of pramlintide added to the apical side (0.01 to 0.25 μM) by 30–44% ($n = 6$, F-value = 5.35; $p < 0.05$).

Effect of amylin on LRP1 expression in bEnd3 cells

To test whether amylin affect A β transport by changing the expression of A β major transport protein, LRP1, which is localized to the basolateral side (brain side) of the *in vitro* BBB model, western blot analysis was performed. As shown in Fig. 7A, although LRP1 expression in the whole lysate was not affected ($n = 4$, t -value = 0.35; $p > 0.05$), amylin increased the expression of LRP1 in the isolated membrane fraction by approximately 1.4-fold ($n = 4$, t -value = 4.63; $p < 0.01$). This result indicated that amylin increased LRP1 trafficking, thus localization, to the membrane domain of the endothelial cells but not the total cellular protein expression. Interestingly, cells treated with the amylin antagonist AC187 diminished amylin ability to enhance LRP1 trafficking to the plasma membrane, reducing its expression level to those of the control (Fig. 7B; $n = 4$, $p < 0.01$).

DISCUSSION

Available studies demonstrated structural similarities in amylin between different species and human with 84% similarities with mouse amylin [41]. Also, the N-terminal and C-terminal amino acid sequences are very highly conserved. Differences in the amino acid sequence of residues 20–29 of amylin have been linked to the higher tendency of human amylin to aggregate that are absent in mice [41]. The human origin amylin and A β peptides share several characteristics including their tendency to aggregate and form similar β -sheet structures [28], degradation by proteases [10], and being able to cross the BBB [42, 43]. Peripherally amylin is mainly produced by the pancreas, and available studies demonstrated amylin is also produced centrally by neurons [44]; however, plasma amylin may cross the BBB to support its brain physiological function, including those related to cognition and memory processing [31]. We have shown previously that a single i.p. injection of amylin provoked an increase in A β blood levels, which raises the possibility that amylin acts through a cognate receptor on the BBB to remove A β from the brain to the blood in AD transgenic animals [31]. Thus, in this follow-up study we hypothesized that amylin alters A β brain to blood clearance by modulating its transport-based mechanism(s) across the BBB. Consistent with our previous findings, a single i.p. injection of amylin also induced a surge of both A β ₄₀ and A β ₄₂ in serum of an AD mouse model, and this increase was abolished by the amylin receptor antagonist AC253 (Fig. 1). *In vitro* studies were then preformed to explain this observation. In these studies, ¹²⁵I-A β ₄₀ was used instead of ¹²⁵I-A β ₄₂ for practicality reasons, as it is less prone to aggregation under the experimental conditions used and has faster clearance rate than A β ₄₂ [35]. Mouse brain endothelial cells treated with amylin showed a concentration-dependent increase in the cellular uptake of ¹²⁵I-A β ₄₀ (Fig. 3). In the transport studies, amylin added to the apical side of the model enhanced the transport of ¹²⁵I-A β ₄₀ from basolateral to apical side (representative for brain to blood, respectively) (Fig. 5), suggesting that the binding of peripheral amylin to its receptor(s) at the luminal side of the endothelial cells enhances brain A β transport. Similarly, brain amylin, centrally produced or from peripheral origin, could also contribute to the transport of brain A β to the blood (Fig. 5B).

All these effects of amylin on A β in the BBB cell model were blocked by the amylin receptor antagonist AC187 and Ramp3 knockdown, suggesting that amylin mediated A β

transport at the BBB via amylin receptors. These results are consistent with the *in vivo* findings and support a role for amylin in mediating A β transport across the BBB (Fig. 1), as the brain is a major source of human A β in this transgenic mouse model [32].

Interestingly, our experiments showed that amylin effect on A β clearance could be attributed to induction of A β transport through the BBB rather than its degradation pathway (Fig. 3C). LRP1 is localized to the brain side of the BBB; it is involved in facilitating A β uptake and subsequent efflux across the BBB [35, 45]. Our own studies have shown previously that induction of LRP1 increased A β clearance from the brain to the blood and also from the liver, the major organ responsible for A β clearance from the periphery [17, 46]. In this study, data from western blot experiment showed that short time treatment (1.5 h) of bEnd3 did not alter LRP1 total protein expression, but significantly induced LRP1 protein expression in the extracted membrane fraction. It suggests that amylin induced subcellular translocation of LRP1 from cytoplasmic compartment to the membrane, and the effect was at the post-transcriptional level because the total protein level of LRP1 from the cell lysate was not affected by amylin treatment (Fig. 7). It is conceivable that increasing the localization of LRP1 at the cell surface would enhance A β transport as more receptors are available for uptake to shuttle A β from basolateral to apical side. Such effect, however, was significantly attenuated when cells were pretreated with AC187 indicating that binding of amylin to its receptor(s) could be responsible for LRP1 trafficking to the membrane by a mechanism yet to be elucidated. A similar effect has been observed with insulin, which showed its ability to facilitate the hepatic uptake of A β by enhancing LRP1 trafficking from intracellular pool to the membrane of hepatocytes [47]. This short-term regulation of LRP1 by amylin at the BBB could influence A β brain levels. Thus, an alteration in amylin blood and/or brain levels due to disease condition or even certain food consumption could interrupt A β brain homeostasis. Furthermore, while there is a possibility for amylin treatment to increase A β transport to the brain from the blood, the localization of LRP1 at the basolateral side of the BBB favors brain to blood transport supported by the *in vivo* and *in vitro* findings. However, whether the effect of amylin on A β enhanced-clearance is mediated only by inducing the translocation of brain endothelial-LRP1 to cell surface or not is not known. Future studies should include LRP1 knockdown in endothelial cells to determine its degree of contribution to amylin effect. Future studies should also investigate the possible effect of amylin on plasma A β of peripheral origin, and whether amylin affects A β clearance through the liver by modulating hepatic LRP1.

Our findings confirmed amylin to cross the endothelial cells of the BBB (Fig. 2) and moves from blood to brain, which is consistent with a previous report [43]. Besides, our data showed for the first time that amylin crosses the BBB from brain to blood as well, however with higher blood to brain transport by approximately 2.5-fold. The beneficial effects of amylin might be surprising to some because prior literature assumed that amylin was a detrimental part of amyloid pathology in AD since amylin deposits have been observed in the pancreas in type 2 diabetic patients [48]. One study noted that presence of amylin associated with amyloid deposits and the cerebrovascular system in AD [49]. However, increasing evidence suggests that while pathological deposits are good diagnostic markers for disease, they are not actually the pathological toxic species [50–52]. In this context, the strong tendency of amylin to aggregate might account for its presence in amyloid deposits,

but does not indicate that amylin is toxic *in vivo*. Indeed, the approval of the amylin analog pramlintide by the FDA combined with its safe and successful use for the treatment of type 2 diabetes emphasizes the clinically beneficial actions of soluble amylin [53, 54]. The pharmaceutical industry optimized the protective actions of amylin by substituting prolines at positions 25, 28 and 29 of human amylin, which prevent amylin from oligomerizing or aggregating [40]. The importance of diabetes as a risk factor for AD [55] highlights yet another potentially beneficial action of the amylin cascade in AD, and this action was examined in the current study. Our data showed that pramlintide exerts a similar effect as amylin to enhance A β removal from brain to blood across the BBB (Fig. 6). Given the favorable safety profile of pramlintide in clinical use, pramlintide should be strongly considered for treatment in AD.

Collectively, the above findings demonstrated that amylin acted through its receptor to stimulate A β brain to blood clearance, at least in part, by inducing the subcellular translocation of LRP1 to the plasma membrane of the BBB endothelial cells, which could explain, at least in part, the increased plasma levels of hA β observed in the current and previous *in vivo* studies [31]. Our study demonstrated a novel mechanism by which amylin enhanced A β brain clearance. Despite its activity of clearing A β from the brain to blood, human amylin is limited to treat AD by a propensity for self-aggregation, thus we propose to use pramlintide, an FDA approved drug for diabetes, for further investigation as a therapeutic agent for AD.

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Fig. 1.

Amylin challenge alters Aβ serum levels and the effect is blocked by an amylin receptor antagonist. Before the challenge experiments with amylin, the mice received a baseline blood draw, and then were treated either with intraperitoneal injection (i.p.) of AC253 or of PBS on daily basis for 3 days ($n = 4$ for each group). 24 h after the third injection, blood draw was conducted for each mouse followed by a single i.p. injection of amylin as a challenge. 24 h after the challenge, the final blood draw was conducted. All blood samples were collected and serum levels of endogenous human Aβ₄₀ (A), or Aβ₄₂ (B) were measured. Data presented as mean $\pm$ S.E.M ($n = 4$, *** $p < 0.001$ compared to pre-injection and PBS treated groups; ns = not significant compared to pre-injection and AC253 injected groups).

Fig. 2.

Amylin transport across the *in vitro* model of BBB. A) Concentration-dependent increase in the transport of amylin, added to the basolateral side, across the *in vitro* BBB model from basolateral to apical (B→A) side. B) Concentration-dependent increase in the transport of amylin, added to the apical side, across the *in vitro* BBB model from apical to basolateral (A→B) side. C) Amylin transport ratio (A→B/B→A). Data presented as mean $\pm$ S.E.M ($n = 5$, ** $p < 0.01$ for amylin transport from A→B compared to B→A).

Fig. 3. bEnd3 cells treatment with amylin enhanced the intracellular levels of A β . A) Cumulative uptake of ^{125}I -A β_{40} in bEnd3 cells after its co-incubation with 0, 5, or 10 μM of amylin. B) Cumulative uptake of ^{125}I -A β_{40} in bEnd3 cells after its co-incubation with of amylin with or without 5 μM of AC187 or 10 μM AC253, amylin receptor antagonists. C) Degradation of ^{125}I -A β_{40} by bEnd3 cells after exposure to amylin 0.05–5 μM for 30 min using TCA precipitation assay. Data presented as mean $\pm$ S.E.M ($n = 6$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant compared to amylin treated cells).

Fig. 4.

Ramp3 knockdown in bEnd3 cells. A) Representative gel image of siRNA knockdown of Ramp3 in bEnd3 cells and bands quantification by densitometric analysis. RT-PCR at 4 h after transfection shows the efficiency of Ramp3 knockdown. M, the DNA size marker. B) Cumulative uptake of ¹²⁵I-Aβ₄₀ in bEnd3 cells after Ramp3 knockdown by siRNA transfection for 4 h followed by amylin treatment. Data presented as mean ± S.E.M (*n* = 3, ***p* < 0.01, ****p* < 0.001, compared to control or amylin treated cells).

Fig. 5.

Addition of amylin to either side of the *in vitro* BBB model enhanced A β transport from basolateral to apical side (B $\rightarrow$ A). Fold increase in transport quotient (TQ_{B $\rightarrow$ A}) of $^{125}\text{I-A}\beta_{40}$, after 45 min treatment with 0.05 to 5 μM of amylin added to apical side (A) or basolateral side (B). C) Effect of amylin (1 μM) added to the apical side with or without AC187 (0.05–5 μM) on TQ_{B $\rightarrow$ A} of $^{125}\text{I-A}\beta_{40}$. Data presented as mean $\pm$ S.E.M ($n = 4$, in A and B data was compared to control; * $p < 0.05$; ** $p < 0.01$; ns = not significant).

Fig. 6.

bEnd3 cells treatment with pramlintide enhanced the intracellular levels of A β . A) Cumulative uptake of $^{125}\text{I-A}\beta_{40}$ in bEnd3 cells after its co-incubation with 0, 0.1, 0.25 or 1.0 μM of pramlintide. B) Fold increase in transport quotient ($\text{TQ}_{\text{B} \rightarrow \text{A}}$) of $^{125}\text{I-A}\beta_{40}$, after 45 min treatment with 0.01 to 0.25 μM of pramlintide added to the apical side. Data presented as mean $\pm$ S.E.M ($n = 6$, * $p < 0.05$, *** $p < 0.001$).

Fig. 7.

Amylin treatment caused LRP1 translocation from cytoplasmic pool to plasma membrane of bEnd3 cells. A) Representative western blots of LRP1 protein from whole cell lysate and membrane isolated protein with their graphical representation of normalized protein expression of LRP1/actin from both total cell lysate or membrane isolated protein. B) Representative western blots showing the AC187 (5 μ M) inhibits amyloid induced translocation of LRP1. Data presented as mean $\pm$ S.E.M ($n = 4$; ** $p < 0.01$, ns = not significant, compared to control).