



# Amyloid- $\beta$ peptides inhibit the expression of AQP4 and glutamate transporter EAAC1 in insulin-treated C6 glioma cells

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## ARTICLE INFO

### Keywords:

Aquaporin 4  
Amyloid  $\beta$   
Glutamate transporter excitatory amino acid carrier 1  
Glutamate uptake  
Alzheimer's disease

## ABSTRACT

Astrocytic aquaporin 4 (AQP4) facilitates glutamate clearance *via* regulation of the glutamate transporter function, involved in the modulation of brain plasticity and cognitive function to prevent neurodegenerative disorders such as Alzheimer's disease (AD). In *in vitro* studies, the C6 rat glioma cell line is a widely applied aging model system to investigate changes in glial cells associated with aging or AD. However, the neurotoxicity mechanism whether AQP4 mediate glutamate uptake in A $\beta$ -stimulated C6 cell remain uncertain. In this study, we examined the effects of A $\beta$  on the expression of AQP4, Glu transporters, Glu uptake, and cell viability in insulin-treated C6 cells. Our results showed that the expression of AQP4 mRNA and protein was significantly enhanced by insulin in older cultures (passage 45), and the expression was inhibited by A $\beta$  at 10  $\mu$ M. In addition, the cell viability and glutamate uptake in A $\beta$ -treated C6 cells were decreased in dose-dependent manners. GFAP showed similar changes in gene and protein expression patterns as AQP4, but no significant alterations were seen in GLAST expression. In C6 cells, the glutamate transport was found to be EAAC1, not GLT-1. EAAC1 expression was decreased by the treatment of A $\beta$ . Taken together, our findings suggest that C6 cells may have astrocytic characteristics, and the astrocytic cytotoxicity induced by A $\beta$  was mediated by reduction of glutamate uptake through AQP4/EAAC1 pathway in C6 cells. This indicates that C6 glioma cells could be used to study the roles of AQP4 on astrocyte function in AD.

## 1. Introduction

Alzheimer's disease (AD) is known as senile dementia, and the formation of amyloid plaques and nerve fiber entanglement is the general cause of AD. However, to date, there are no molecular targets to prevent the onset of AD and disease progression [1–5].

Glutamate (Glu) is an excitatory neurotransmitter essential for synaptic plasticity, excitatory synaptic transmission, and neuronal development. However, excessive Glu accumulation in synaptic clefts is known to cause neurodegenerative diseases such as ischemia, epilepsy and amyotrophic lateral sclerosis [6]. Thus, regulation of Glu outside the cells is essential for excitotoxicity prevention and normal synaptic transmission. Glu transporter is the primary mechanism for removal of the released Glu. Rothstein et al. [7] revealed that both neurons and astrocytes can act as high affinity Glu transporters. Glu levels outside the astrocyte or glial cells are maintained at low concentrations by Glu transporters *via* activation of both neurons and glial cells. Glu

transporters, such as glutamate transporter 1 (GLT-1), glutamate aspartate transporter (GLAST), and excitatory amino acid transporter 1 (EAAC1), are widely distributed throughout the CNS [8–10]. These transporters play important roles as glutamate, and are thought to modulate the formation and elimination of synapses as well as neuronal migration, proliferation and apoptosis [11]. In addition, Glu transporters including EAAC1, GLAST and GLT-1 in rats and rabbits are localized to the astrocytes and neurons [7,12]. GLAST and GLT-1 are expressed in the astrocyte, whereas EAAC1 and GLAST are expressed in the glial cells [13–15]. Also,  $\beta$ -amyloid (A $\beta$ ) accumulation outside the glial cells, astrocytes, and neurons is revealed the outcome of Glu transporter dysfunction [16].

Aquaporins (AQPs) are a group of water-selective membrane transport channels that allow rapid transfer of water. In the normal brain, aquaporin-1 and aquaporin-4 (AQP4) are the most studied, also AQP4 is a major isoform of AQPs in the adult brain. It is mainly expressed in astrocytes of the central nervous system, and AQP4 is well known to

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<https://doi.org/10.1016/j.toxrep.2020.08.032>

Received 2 September 2019; Received in revised form 9 August 2020; Accepted 27 August 2020

Available online 31 August 2020

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support the regulation of astrocytic functions. Several studies have revealed that AQP4 plays roles in synaptic plasticity, contributing to memory by regulating the Glu transporter expression. Recent studies have revealed that AQP4 knockout or downregulation exacerbates brain fluid and ion homeostasis [17–20], is involved in cell migration and neuronal scar formation [17,21], and is associated with astrocytic functions such as neurotransmission and synaptic plastic deformation [22,23]. Furthermore, it is reported to cause a disorder resulting in the secretion of the inflammation inducer [24]. Thus, AQP4 is being investigated not only for mental disorders including depression, but also for various diseases of the nervous system [25–28].

Glial cells are known to regulate A $\beta$  accumulation through AQP4 expression *in vivo*. However, the results of current *in vitro* studies on the expression of AQP4 in glial cell line are unclear. Capoccia et al. [24] reported that the AQP4 inhibition in C6 cells affects cell migration and apoptosis. Contrarily, Dolman et al. [25] and Yoneda et al. [26] found that the C6 cells were unable to show the AQP4 expression.

Insulin is widely expressed in the brain, and increases astrocytic expression in cultures of glial cells. Goya et al. [28] reported that insulin treatment promotes C6 glioma cell differentiation, thereby having astrocytic properties by activating enzyme activity of glutamine synthetase, an astrocyte biomarker. Therefore, insulin had been used as a differentiation agent for realizing the properties of astrocytes in C6 glioma cells in this study.

In astrocytes, decreasing level of GFAP leads to the activation of caspase-3 and cell damage, which are common features in AD [29,30]. In addition, Varmazyari et al. [31] revealed that GFAP determination of cortex and cerebellum in rats inhibited by neuronal toxicity. Treatment with A $\beta$  peptides results in apoptosis of astrocytes or neurons, with the subsequent activation of cleaved caspase-3. The activation of cleaved caspase-3 caused by apoptosis of A $\beta$  peptides-treated astrocyte or neuron. Zhang et al. [32] reported that activation of Bax and inhibition of bcl-2 induces Cytochrome C which, in turn, commits the cell to apoptosis by activating the release of caspase related proteins.

In this study, we investigate the expression of AQP4 in response to A $\beta$  in the presence of insulin as a differentiation promoting agent. In the insulin-treated C6 cells, effects of A $\beta$  peptide on the expression of AQP4, Glu transporters, Glu uptake, and cell viability were examined to observe the changes in astrocytic responses in A $\beta$ -stimulated C6 glioma cells.

## 2. Materials and methods

### 2.1. Reagents

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), insulin, wortmannin were purchased from Sigma-Aldrich (St. Louis, MO). Antibody against AQP4 (sc-32739) was purchased from Santa Cruz Biotechnology (California, USA), cleaved caspase-3 (#9664), GLT-1 (#3838), GLAST (#5684), EAAC1 (#14501), GFAP (#80788), and horseradish peroxidase-linked anti-mouse secondary antibody (#7076), horseradish peroxidase-linked anti-rabbit secondary antibody (#7074) were purchased from Cell Signaling Technology (Beverly, MA, USA). The  $\beta$ -amyloid<sub>25-35</sub> (A $\beta$ <sub>25-35</sub>) and  $\beta$ -amyloid<sub>1-42</sub> (A $\beta$ <sub>1-42</sub>) were purchased from Abcam Biotechnology (Cambridge, UK). TGN-020 was purchased from Tocris Bioscience (Bristol, United Kingdom). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) were purchased from the American Type Culture Collection (Manassas, VA, USA).

### 2.2. Cell culture

C6 cells were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM supplemented with penicillin (120 units/mL), streptomycin (120 units/mL), and 10 % FBS in a 5 % CO<sub>2</sub> atmosphere at 37 °C.

### 2.3. Reverse transcription polymerase chain reaction (RT-PCR)

C6 cells were seeded into a 6-well cell culture plate ( $1 \times 10^5$  cells per well, passage number 45) for 24 h. And the aggregated A $\beta$ <sub>1-42</sub> or A $\beta$ <sub>25-35</sub> (0.1, 0.5, 1, 5 or 10  $\mu$ M) with insulin (10 ng/mL) treated with C6 cells for 48 h. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The RNA purity and concentration was measured by spectrometry at 260, 280 nm. Total RNA was reverse transcribed into cDNA using a SuperScript® II Reverse Transcriptase (18064-014, Thermo Fisher Scientific, Inc., Waltham, USA) according to the manufacturer's protocol. The amplification conditions were as follows: AQP4, 45 s at 98 °C followed by 30 cycles of 10 s at 98 °C, 30 s at 57 °C and 60 s at 72 °C; GLT-1, 45 s at 98 °C followed by 30 cycles of 10 s at 98 °C, 30 s at 55 °C and 60 s at 72 °C; GLAST, 45 s at 98 °C followed by 30 cycles of 10 s at 98 °C, 30 s at 60 °C and 60 s at 72 °C; EAAC1, 45 s at 98 °C followed by 30 cycles of 10 s at 98 °C, 30 s at 54 °C and 60 s at 72 °C; GFAP, 45 s at 98 °C followed by 30 cycles of 10 s at 98 °C, 30 s at 60 °C and 60 s at 72 °C;  $\beta$ -actin, 45 s at 98 °C followed by 30 cycles of 10 s at 98 °C, 30 s at 58 °C and 60 s at 72 °C. Primers sequences were as follows: AQP4 forward, 5'-GC ATG AAT CCA GCT CGA TCC TTT GG-3' and reverse, 5'-AA TGG GTG GCA GGA AAT CTG AGG C3' (product, 315 bp); GLT-1 forward, 5'-TAC AGC CCT TTA CGA AGC C-3' and reverse, 5'-TGA TAG ACA ATC CCA GCC C-3' (product, 242 bp); GLAST forward, 5'-CTA CTC A CCG TCA CCG CTG T-3' and reverse, 5'-AGC ACA AAT CTG GTG ATG CG-3' (product, 1012 bp); EAAC1 forward, 5'-TGT TAG TTG TGA GCA TCA AGC-3' and reverse, 5'-CCT TTT TCT CCC ATT TTT CC-3' (product, 324 bp); GFAP forward, 5'-GGT GTC CAG GCT GGT TTC TC-3' and reverse, 5'-CAA GCC AGA CCT CAC AGC G-3' (product, 515 bp);  $\beta$ -actin forward, 5'-GA GGC ATC CTG ACC CTG AAG-3' and reverse, 5'-CA TCA CAA TGC CAG TGG TAC G-3' (product, 433 bp). Differential expression was calculated by analysis of the target gene amplification following normalization to the rat  $\beta$ -actin endogenous reference.

### 2.4. Western blot

C6 cells were seeded into a 6-well cell culture plate ( $1 \times 10^5$  cells per well, passage number 45) for 24 h, and then treated with aggregated A $\beta$ <sub>1-42</sub> or A $\beta$ <sub>25-35</sub> (0.1, 0.5, 1, 5 or 10  $\mu$ M) or TGN-020 (AQP4 inhibitor, 1  $\mu$ M) or wortmannin (EAAC1 inhibitor, 1  $\mu$ M) with insulin (10 ng/mL) for 48 h. C6 cells were lysed in membrane protein extraction kit (Thermo Fisher Scientific, Inc., Waltham, USA). Membrane protein concentrations were measured using a BCA protein assay (Thermo Fisher Scientific, Waltham, USA). Membrane proteins (100  $\mu$ g) were separated by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to PVDF membranes (Whatman GmbH, Dassel, Germany). The transferred proteins blocked with 5% skimmed milk in Tris-buffered saline containing 0.1 % Tween-20 for 1 h and then incubated with AQP4 (1:200 dilution), cleaved caspase-3 (1:500 dilution), GLT-1 (1:200 dilution), GLAST (1:200 dilution), EAAC1 (1:200 dilution), GFAP (1:200 dilution),  $\beta$ -actin (1:1,000 dilution) for overnight at 4 °C. After three washes in Tris-buffered saline containing 0.1 % Tween-20, membranes were incubated with horseradish peroxidase-linked anti-mouse secondary antibody for 1 h. Proteins were detected by enhanced chemiluminescence and visualized using image software (UVP Vision Works® LS Image Acquisition & Analysis Software, Upland, CA).

### 2.5. Cell viability assay

The A $\beta$ -treated C6 cell viabilities were measured using MTT colorimetric assay. Briefly, C6 cells ( $5 \times 10^4$  cells per well, passage number 45) were seeded in a 24-well cell culture plate. And the aggregated A $\beta$ <sub>1-42</sub> or A $\beta$ <sub>25-35</sub> (0.1, 0.5, 1, 5 or 10  $\mu$ M) with insulin (10 ng/mL) were treated with seeded C6 cells for 48 h. The produced formazan crystals were dissolved in 500  $\mu$ L of DMSO, and absorbances were measured by spectrophotometry using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at 550 nm.

## 2.6. Glutamate uptake

Glu uptake was studied in C6 cells seeded at  $1 \times 10^5$  cells per 6-well cell culture plate (passage number 45). After 24 h incubation, the cells were treated with various concentrations of aggregated A $\beta_{1-42}$  or A $\beta_{25-35}$  (0.1, 0.5, 1, 5, 10  $\mu$ M) with insulin (10 ng/mL). After 48 h incubation, the cells lysates were prepared and luciferase activity was determined using the Glutamate-Glo™ assay (TM495, Promega Corporation, Madison, Wisconsin, USA) and luminescence determined using a luminometer (GM2000, Promega Corporation, Madison, Wisconsin, USA).

## 2.7. Statistical analysis

Results are presented as means  $\pm$  standard deviations ellipse (SDE) values of three experiments. A significant difference from the respective control for each experimental test condition was assessed using Student's *t*-test for each paired experiment, and significant difference is considered at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ .

## 3. Results

### 3.1. Effects on AQP4 levels in insulin and A $\beta$ and insulin-treated C6 cells

Changes in AQP4 levels were investigated in insulin-treated C6 cells (passage 15, 28, 45). The normal early (passage 18, 28) or elder (passage 45) C6 cells showed weak AQP4 protein expression (Fig. 1A, lane 1, 3, 5). However, exposure of the elder C6 cells to insulin enhanced the AQP4 protein expression about 1.6-fold (Fig. 1A, lane 5, 6). A $\beta_{1-42}$  or A $\beta_{25-35}$  of lower concentration increased the AQP4 levels (5.04-fold at 0.1  $\mu$ M, 4.37-fold at 0.5  $\mu$ M, respectively); however, suppression of AQP4 expression was observed after exposure to high concentration (10  $\mu$ M) of A $\beta_{1-42}$  (0.75-fold) and A $\beta_{25-35}$  (0.86-fold) (Fig. 1B). In addition, the AQP4 mRNA expression in A $\beta_{1-42}$  (0.1  $\mu$ M) or A $\beta_{25-35}$  (0.5  $\mu$ M)-treated C6 cells was up-regulated about 1.6-fold compared to the control cells (Fig. 1C), but at 10  $\mu$ M A $\beta$  peptides, the AQP4 mRNA expressions were significantly decreased.

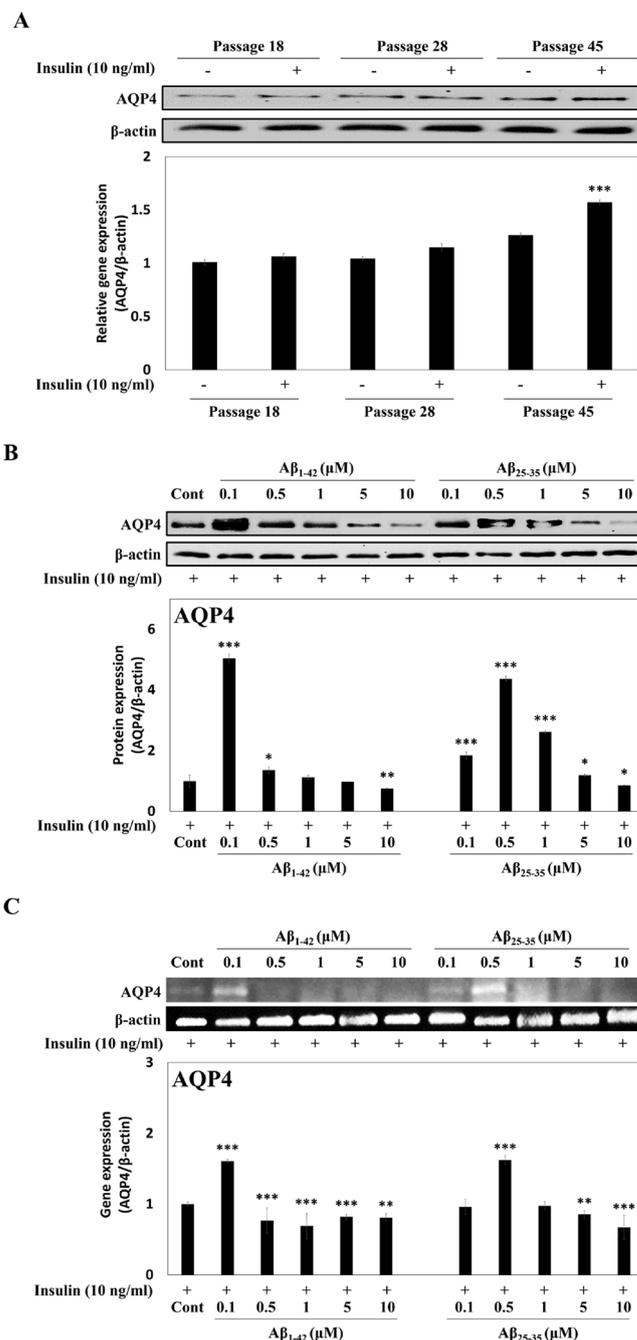
### 3.2. Effect of A $\beta_{1-42}$ or A $\beta_{25-35}$ on the cytotoxicity and glutamate uptake in insulin-treated C6 cells

A $\beta_{1-42}$  or A $\beta_{25-35}$  reduced the cell viability up to 48.4 % or 55.1 % at the concentration of 10  $\mu$ M in insulin-treated C6 cells (passage number 45) (Fig. 2A). Under the same condition, Glu uptake in the cells (passage number 45) was also inhibited by the A $\beta$  peptides in a dose-dependent manner (Fig. 2B), suggesting that A $\beta$  induced accumulation of Glu and is assumed to cause cell death.

Caspase-3 is considered to be a phenotype of apoptosis, and is responsible for the maturation of procaspase-3. Cleavages of caspase-3 substrates are reported to play a vital role in neuronal apoptosis [25]. We therefore performed Western blot analysis to examine the effect of A $\beta$  peptide treatment on caspase-3 protein expression in insulin-treated C6 cells (passage number 45). Exposure of insulin-treated C6 cells to A $\beta_{1-42}$  (0.1–10  $\mu$ M) dose-dependently upregulated the cleaved caspase-3 by approximately 1.45-, 1.69-, 2.08-, 2.43-, and 2.65-fold, respectively, as compared to the insulin-treated C6 cells (Fig. 2C). Moreover, A $\beta_{25-35}$  exposure (0.1–10  $\mu$ M) also promoted the dose-dependent increase of cleaved caspase-3 protein expression by 1.16-, 1.36-, 1.88-, 2.30-, and 2.57-fold, respectively, as compared to insulin-treated C6 cells. Our data therefore confirms that apoptotic cell death is the outcome of A $\beta$  accumulation or targeted A $\beta$  sequence part in C6 cells.

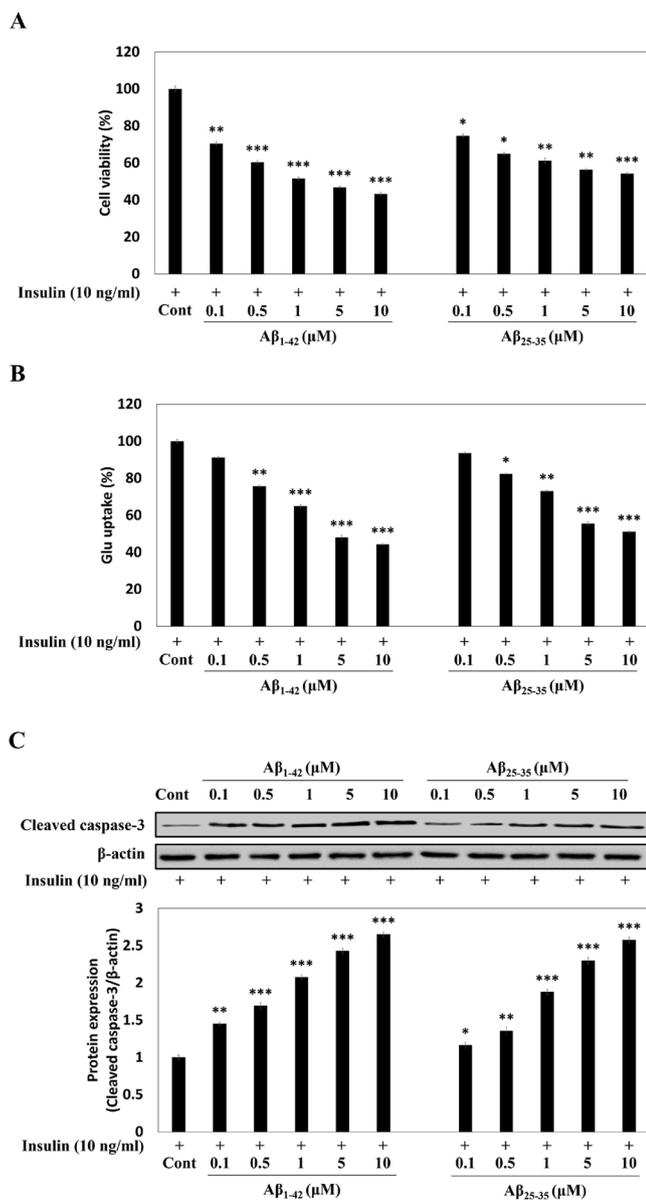
### 3.3. Effects of A $\beta_{1-42}$ or A $\beta_{25-35}$ on the expressions of Glu receptors and GFAP in insulin-treated C6 cells

We then checked the changes in protein expressions of Glu receptors



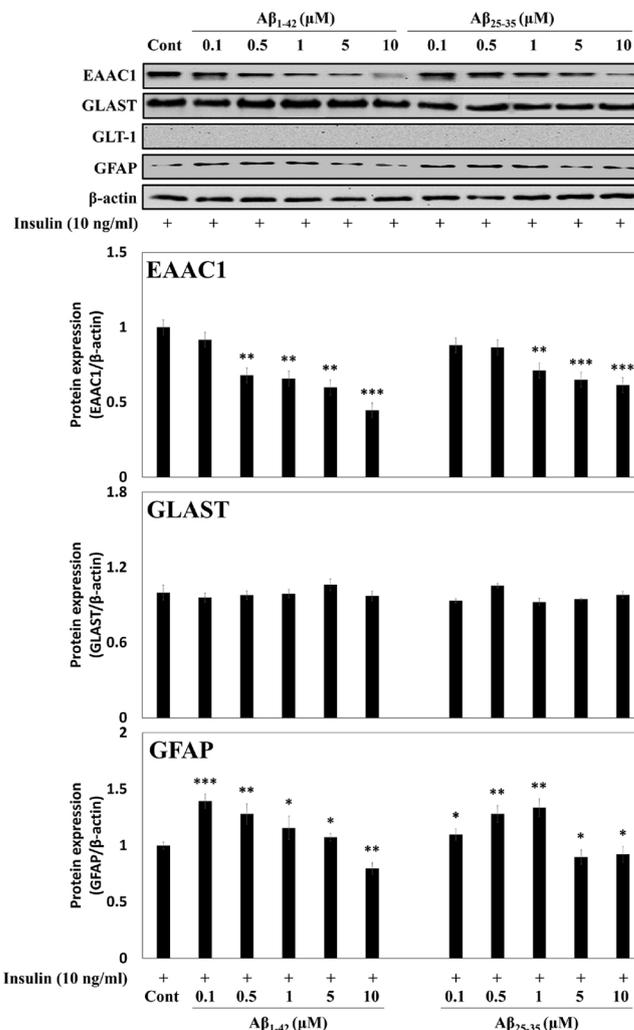
**Fig. 1.** AQP4 expressions in C6 cells under various conditions, (A) effects of cell passages and insulin on the AQP4 protein expression, (B) the AQP4 protein expression in A $\beta_{1-42}$  or A $\beta_{25-35}$ -treated C6 cells in the presence of insulin, (C) the AQP4 gene expression in A $\beta_{1-42}$  or A $\beta_{25-35}$ -treated C6 cells in the presence of insulin.  $\beta$ -Actin was used as the internal control for Western blot and RT-PCR analysis. Results are presented as the means  $\pm$  SDE of percentages calculated with respect to control levels of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. insulin alone ( $n = 3$ ).

and GFAP upon exposure to A $\beta$  by Western blot. Since the expression of GLUT-1 mRNA and protein was not detected in insulin-treated C6 cells (passage number 45), it was not examined further. Exposure to A $\beta_{1-42}$  or A $\beta_{25-35}$  suppressed the EAAC1 protein expression about 0.4-fold or 0.6-fold, respectively, at 10  $\mu$ M compared with the control, suggesting that the inhibition of Glu uptake in A $\beta$ -treated C6 cells might be mediated by the suppression of the EAAC1 expression (Fig. 3). GFAP expression was also significantly reduced about 0.8 and 0.9-fold at 10  $\mu$ M of A $\beta$ , respectively.



**Fig. 2.** Effects of Aβ peptides on the cell viability (A), Glu uptake (B), and caspase-3 protein expression (C), in insulin-treated C6 cells (passage number 45). TGN-020 was used as an AQP4 inhibitor, and wortmannin used as an EAAC1 inhibitor in the insulin-treated C6 cells. Results are presented as the means ± SDE of percentages calculated with respect to control levels of three independent experiments. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 vs. insulin alone (*n* = 3).

In addition, the gene expressions of the Glu receptors and GFAP in Aβ<sub>1-42</sub> or Aβ<sub>25-35</sub> with insulin-treated C6 cells (passage number 45) were measured using RT-PCR. As with the protein expressions, mRNA expression of EAAC1 was reduced by 0.7-fold and 0.6-fold at 10 μM, respectively, with the treatment of Aβ<sub>1-42</sub> or Aβ<sub>25-35</sub>, while GLAST showed no change in the expression. On the other hand, GFAP expression was also significantly reduced about 0.7 and 0.6-fold at 10 μM of Aβ, respectively (Fig. 4). From these results, it was confirmed that insulin treatment upregulated the expression of AQP4 in insulin-treated C6 cells, which was inhibited by exposure to Aβ peptides. Overall, both Aβ peptides exhibited similar effects, but in particular, Aβ<sub>1-42</sub> showed significant inhibitory effects on the cell viability, Glu uptake, and the expressions of Glu transporters and GFAP at concentration of 10 μM.



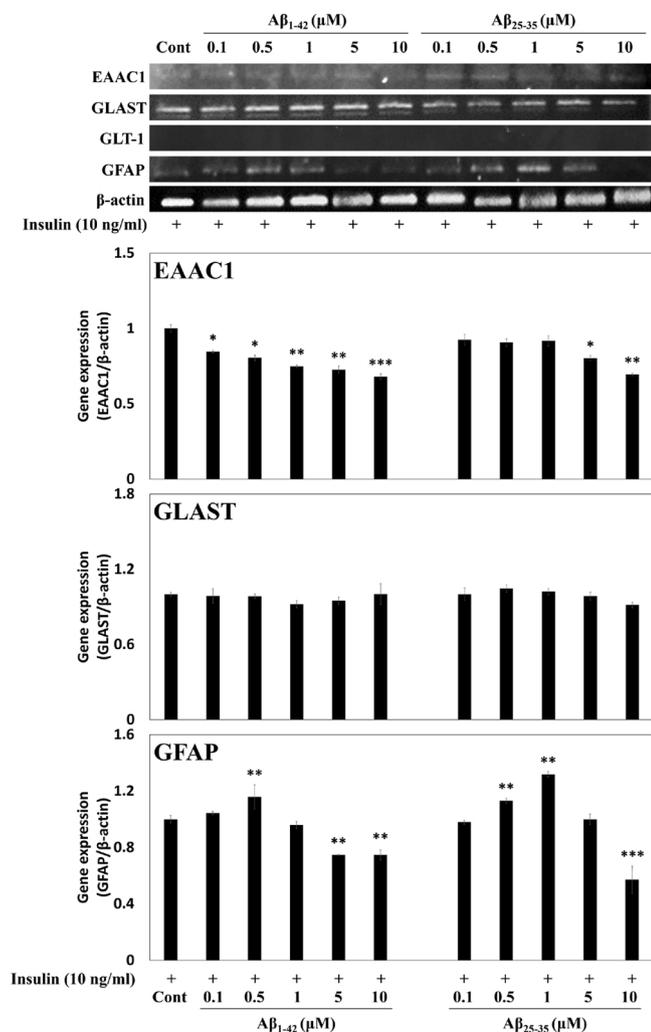
**Fig. 3.** Effects of Aβ peptides on Glu receptors and GFAP protein expressions in insulin-treated C6 cells (passage number 45). β-Actin was used as the internal control for Western blot analysis. Results are presented as the means ± SDE of percentages calculated with respect to control levels of three independent experiments. Results are presented as the means ± SDE of percentages calculated with respect to control levels of three independent experiments. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 vs. insulin alone (*n* = 3).

**3.4. Effect of relationship between AQP4, GFAP, EAAC1 in insulin-treated C6 cells**

AQP4 is co-expressed with glutamate transporters, and acts as a selective transmembrane pore for water transport [14]. In addition, the accumulation of Aβ peptides in astrocytes results in structural destruction of the astrocyte [16]. In this study, we evaluated protein expressions of AQP4, GFAP, and EAAC1 after exposure to TGN-020 (AQP4 inhibitor, 10 μM) or wortmannin (EAAC1 inhibitor, 0.1 μM), in insulin-treated C6 cells (passage number 45). Inhibition of AQP4 was observed to suppress the protein expressions of AQP4, EAAC1, and GFAP by 0.49-, 0.39-, and 0.50-fold, respectively, as compared to the control insulin-treated C6 cells (Fig. 5). However, inhibition of EAAC1 decreased EAAC1 and GFAP protein expressions by about 0.35- and 0.85-fold, respectively, but not AQP4 protein expression, when compared to the insulin-treated C6 cells. Our data indicates that AQP4 expression is upstream of EAAC1 and GFAP protein expressions.

**4. Discussion**

AQP4, which is present in astrocytes, has been shown to play an



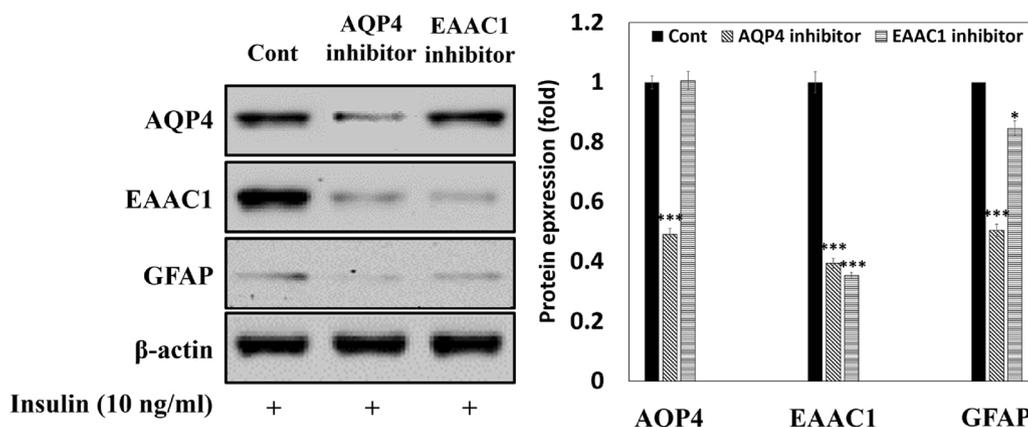
**Fig. 4.** Effects of Aβ peptides on Glu receptors and GFAP gene expressions in insulin-treated C6 cells (passage number 45). β-Actin was used as the internal control for RT-PCR analysis. Results are presented as the means ± SDE of percentages calculated with respect to control levels of three independent experiments. Results are presented as the means ± SDE of percentages calculated with respect to control levels of three independent experiments. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 vs. insulin alone (*n* = 3).

important role in the regulation of memory and cognitive function by mediating the clearance of amyloid to reduce cytotoxicity and thereby protecting neurons. Several studies have reported that deficiency of AQP4 is associated with memory impairment [27]. It was found that the brain in long term of AQP4 gene knockout mice increased brain Aβ plaque deposit compared to normal mice, and also, the behavioral study of AQP4 knockout mice showed impairment in memory [33]. Moreover, astrocytes and microglia play central roles as regulators for Aβ clearance and degradation [34–36]. However, the relationship between AQP4 and Aβ-induced toxicity in C6 cells, an astrocytic cell line, has not been explored previously. In the present study, we investigated whether the expressions of AQP4 and Glu transporter together with cytotoxicity and Glu accumulation in C6 cells are regulated by Aβ peptides for the first time. AQP4 protein was expressed in C6 cells, but the level was very low and it was confirmed that the expression increased as the number of cell passage increased (Fig. 1A). Our results indicate that the later cell passage shows more astrocytic properties, which is consistent with the previous reports [28,37].

To study the role of astrocytic AQP4 in C6 cells, optimum conditions for its expression showing astrocytic properties were examined. Our data revealed that insulin promoted AQP4 expression in C6 cells, as previously suggesting insulin as a differentiating agent for astrocytic cells from dendrocytic C6 cells.

The expression of AQP4 with Aβ treatment (Aβ<sub>1-42</sub> and Aβ<sub>25-35</sub>) were investigated in astrocytic C6 cells in this study. Recent study using cultured cortical neurons demonstrated that Aβ<sub>25-35</sub> or Aβ<sub>1-42</sub> induces cell damage by increasing the Glu release [38]. Lower concentration of Aβ<sub>1-42</sub> or Aβ<sub>25-35</sub> (up to 0.1 μM and 0.5 μM, respectively) results in over-expression of AQP4, but Aβ<sub>1-42</sub> or Aβ<sub>25-35</sub> treatments at higher concentration (5 μM and 10 μM, respectively), results in suppression of AQP4. In addition, our data confirmed that the change in expression of AQP4 by treatment with the Aβ<sub>1-42</sub> peptide was more sensitive than the Aβ<sub>25-35</sub> peptide (Fig. 1B). Yang et al. [29] tested the effect of AQP4 on astrocyte damage after incubating with various concentrations of Aβ<sub>1-42</sub> (0.1, 1 and 10 μM); these data revealed that exposure to AQP4 decreases the astrocyte damage induced by Aβ<sub>1-42</sub> or Aβ<sub>25-35</sub>. In our data, Aβ peptides, which decreased AQP4 expression, induced cell death, suggesting that AQP4 may regulate the Aβ-induced toxicity in C6 cells to protect astrocytic function.

We examined that the expression of Glu receptors in insulin and Aβ-treated C6 cells. EAAC1 expressions were down-regulated by Aβ<sub>1-42</sub> or Aβ<sub>25-35</sub> treatment, which induced decrease in Glu uptake, suggesting that EAAC1 is a major Glu transporter in C6 cells. Likewise, Dall’Igna et al. [39] revealed that the C6 cells express EAAC1, which in turn regulates Glu uptake. On the other hand, GLT-1 is not expressed in glial cells, but is expressed only in astrocytes, which are inhibited by Aβ



**Fig. 5.** Relationship between AQP4, EAAC1, and GFAP protein expressions in insulin-treated C6 cells (passage number 45). TGN-020 and wortmannin were used as AQP4 inhibitor and EAAC1 inhibitor, respectively, in insulin-treated C6 cells. Results are presented as the means ± SDE of percentages calculated with respect to control levels of three independent experiments. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 vs. insulin alone (*n* = 3).

through MAPK pathway and oxidative stress [13–17].

GFAP is an intermediate microfibrous protein of astrocytes, and expression of GFAP is used as a marker of astrogliosis regulation. Accumulation of A $\beta$  plaques from astrocytes is accompanied with a decrease in GFAP expression [29]. From our data, GFAP and AQP4 expressions in C6 cells are suppressed by A $\beta$  treatment at higher concentration, suggesting that A $\beta$ -induced cell damage is due to astrocytes dysfunction through the inhibition of AQP4. In other words, it seems that A $\beta$  clearance is mediated by the expression of AQP4 and Glu transporter in C6 cells as revealed in astrocytes [40,41].

In this study, we confirmed the altered expressions of AQP4 and EAAC1, and the mechanism of the AQP4/EAAC1 pathway protecting C6 cells from damage by A $\beta$ . In order to establish an AD model in a C6 cell, the influence of cell passage and A $\beta$  peptides concentration were investigated, and an *in vitro* model with astrocyte characteristics was implemented by inducing the differentiation of C6 cells by insulin treatment. This study is expected to be able to study the association of AQP4 expression to natural products such as Panax ginseng [42] or *Scrophularia buergeriana* extract [43] reported as AD prophylactic agents using C6 cells with astrocyte properties [44]. We believe that our data will facilitate further studies on the astrocytic functions targeting AQP4 in AD.

#### CRedit authorship contribution statement

**Se-Ho Park:** Methodology, Investigation, Validation, Data curation, Writing - original draft, Visualization. **Jaе-Yeul Lee:** Software, Resources, Formal analysis. **Kwang-Hwan Jhee:** Writing - review & editing. **Seun-Ah Yang:** Conceptualization, Supervision, Project administration, Funding acquisition.

#### Declaration of Competing Interest

The authors report no declarations of interest.

#### Acknowledgment

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2018R1D1A1A02048746).

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