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NEUROPROTECTIVE EFFECT OF 3,5-DI-O-CAFFEOYLQUINIC ACID ON SH-SY5Y CELLS AND SAMP8 MICE THROUGH THE UP-REGULATION OF PGK1

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Abbreviations: Aβ, β-amyloid protein; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AO, antisense oligonucleotide; ATCC, American Type Culture Collection; BBB, blood–brain barrier; CA, caffeoylquinic acid; CBB, coomassie brilliant blue; CQA, caffeoylquinic acid; IEF, isoelectric focusing; MALDI-ToF, matrix assisted laser desorption ionization-time of flight; MWM, Morris water maze; SAMP, senescence-accelerated-prone mice; SAMR, senescence-accelerated-resistant mice; PGK1, phosphoglycerate kinase-1; THP, tetrahydropapaveroline.

Abstract-As aged population dramatically increases in these decades, efforts should be made on the intervention for curing age-associated neurologic degenerative diseases such as Alzheimer's disease (AD). Caffeoylquinic acid (CQA), an antioxidant component and its derivatives are natural functional compounds isolated from a variety of plants. In this study, we determined the neuroprotective effect of 3,5-di-O-CQA on $A\beta_{1-42}$ treated SH-SY5Y cells using MTT assay. To investigate the possible neuroprotective mechanism of 3,5-di-O-CQA, we performed proteomics analysis, real-time PCR analysis and measurement of the intracellular ATP level. In addition, we carried out the measurement of escape latency time to find the hidden platform in Morris water maze (MWM), real-time PCR using senescence-accelerated-prone mice (SAMP) 8 and senescence-accelerated-resistant mice (SAMR) 1 mice. Results showed that 3,5-di-O-CQA had neuroprotective effect on $A\beta_{1-42}$ treated cells. The mRNA expression of glycolytic enzyme (phosphoglycerate kinase-1; PGK1) and intracellular ATP level were increased in 3,5-di-O-CQA treated SH-SY5Y cells. We also found that 3,5-di-O-CQA administration induced the improvement of spatial learning and memory on SAMP8 mice, and the overexpression of PGK1 mRNA. These findings suggest that 3,5-di-O-CQA has a neuroprotective effect on neuron through the upregulation of PGK1 expression and ATP production activation.

Keywords: 3,5-di-O-caffeoylquinic acid, SH-SY5Y, SAMP8, PGK1, Glycolysis, ATP

The major reason of aging is the functional decrease of energy production in the body. Aging is usually associated with the accumulation of reactive oxygen species (ROS)-mediated oxidative damage, which can induce the decrease in intracellular ATP levels via the functional decline of mitochondrion. Intracellular ATP depletion causes cell death in neuronal cells, leading to neurodegenerative diseases such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and Parkinson's disease (Beal et al., 1995; Huaug et al., 2001). AD, in particular, is one of the diseases with increasing number of patients with age-related neurodegenerative disorders, and is a degenerative disorder of the central nervous system, which causes mental deterioration and progressive dementia. AD is accompanied by neuropathologic lesions including the presence of senile plaques of the β-amyloid protein (Aβ), an etiological role in AD. The overexpression of Aβ protein and its fibrillar deposition in senile plaques have been correlated with the progression of cognitive impairment in AD (Eckert et al., 2003; Roth, 2001; Holliday, 1996).

One of the animal models that is used to study AD and aging is the senescence-accelerated mouse (SAM). The SAM model was developed in 1981, which originally consisted of nine major senescence-accelerated-prone mice (SAMP) substrains and three major senescence-accelerated-resistant mice (SAMR) substrains, each of which exhibits the characteristic disorders. Thereafter, selective inbreeding was applied based on the degree of senescence, the lifespan, and the age-associated pathologic phenotypes (Hosokawa et al., 1997; Takeda et al., 1981). The SAMP8 strain exhibits age-related deterioration in memory and learning (Yagi et al., 1988; Ohta et al., 1989) along with the overexpression of amyloid precursor protein (APP) (Li et al., 2009; Nomura et al., 1996; Morley et al., 2000). SAMP8

mice also show decreased glucose metabolism (Poon et al., 2005; Shimano, 1998), which is a characteristic of AD wherein energy metabolism is impaired (Blass et al., 1988). The decrease in the production of Aβ, after giving an intracerebroventricular injection of a 42-mer phosphorothiolated antisense oligonucleotide (AO) directed at the Aβ region of the APP gene, can reduce lipid peroxidation and protein oxidation and improve cognitive deficits in aged SAMP8 mice. Therefore, SAMP8 is a good model to study brain aging and is used as one mouse model of AD.

Caffeoylquinic acid (CQA) derivatives are natural functional compounds isolated from a variety of plants and possess a broad range of pharmacological properties, including antioxidant, hepatoprotectant, antibacterial, antihistaminic, anticancer, and other biological effects (Basnet *et al.*, 1996; Kwon *et al.*, 2000; Nakajima et al., 2005). Recently, it has been demonstrated that CQA derivatives possess neuroprotective effects in Aβ-induced PC12 cell toxicity (Hur *et al.*, 2001) and in tetrahydropapaveroline (THP)-induced C6 glioma cell death (Soh *et al.*, 2003). Moreover, CQA exhibited a neuroprotective function against *in vitro* cell death and *in vivo* ischemia-induced neuronal damage. However, the mechanism by which these caffeoylquinic acid derivatives exert neuroprotection is unclear.

In this study, we used a SH-SY5Y human neuroblastoma cell line as the Aβ-induced neuronal cell death model and the SAMP8 mice as the *in vivo* AD model, to determine a pharmacological function for the the neuroprotective properties of 3,5-di CQA. Furthermore, to investigate the mechanism for the neuroprotective effect of 3,5-di CQA, we have performed (2D)-polyacrylamide-gel electrophoresis (PAGE), matrix assisted laser

desorption ionization-time of flight (MALDI-ToF) mass spectrometry analysis and real time PCR.

EXPERIMENTAL PROCEDURRS

Cell culture

The human neuroblastoma clonal SH-SY5Y cell line was obtained from American Type Culture Collection (ATCC). Cultures were maintained in serum-containing medium (1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12), supplemented with 15% fetal bovine serum and MEM non-essential amino acids. Cells were maintained at 37°C under 5% CO_2 / 95% air. To culture for the subsequent extraction of protein and total RNA, cells were seeded onto Petri plates at a density of 2 x 10^6 cells per dish, and 3,5-di-O-CQA was added at a final concentration of 20 μM.

Determination of cell viability

To investigate the neuroprotective effect, we employed the Aβ-treated SH-SY5Y cell model (Li et al., 1996; Wang *et al.*, 2009). Cell viability was assessed using the conventional MTT reduction assay. The cultured cells in 96-well plates (fibronectin coated plate) were treated with 3,5-di-O-CQA and exposed to 2 μM $A\beta_{1-42}$ for 72 h, then 10 μl of MTT stock solution (5 mg/ml) was added to the culture medium and incubated for 6 h at 37 °C. The formazan was extracted with 100 μl 10% SDS (W/V) and the absorbance was measured with a microtiter plate reader.

Two-dimensional gel electrophoresis (2-DE)

2-DE was performed essentially as described by Isoda et al. (2006) with modifications. Samples containing 30 μg for analytical gels or 300 μg for preparative gels of protein were separated by isoelectric focusing (IEF) and then by sodium dodecyl sulfate polyacrylamide gel eletrophoresis (SDS-PAGE) using Ettan IPGphor II and Ettan DALTsix (GE Healthcare, Uppsala, Sweden). For IEF, samples were added into the rehydration solution containing 8 M urea, 2% CHAPS, 0.5% IPG buffer, 0.002% bromophenol blue and 0.28% (w/v) dithiothreitol (DTT), and then applied to a 24cm immobiline dry strips, which are dry polyacrylamide gel strips with an immobilized pH gradient pH 3-10 (GE Healthcare). The dry strips were rehydrated at 20 ℃ for 12 h and isoelectric focusing of proteins was carried out at 500 V for 1 h, 1000 V for 1 h, 10000 V for 3 h, 10000 V for 2 h, and, 45 min. Thereafter, IPG strips were reduced (1% DTT) and alkylated (2.5% iodoacetamide) in equilibration buffer (6 M urea, 50 mM Tris-Cl, pH 8.8, 30% glycerol, 2% SDS). When the equilibration was finished, the strips were loaded onto 12% acrylamide vertical gels and separation of proteins with different molecular weight was carried out at 2.5 W per gel for 30 min, followed by 25 W per gel for 3.5 h.

Silver staining using Plus One Silver Staining Kit (GE Healthcare) was performed according to the manufacturer's instructions. The stained gels were subjected to image analysis by ImageMaster 2D Platinum software (ver. 4.9; GE Healthcare). Gels in which proteins from either β-amyloid (Aβ) or caffeoylquinic acid (CA) treatment, or both, as well as control were imported into the same platform and were subjected to image analysis. After spot detection and matching, the spots of interest were manually selected and the

data regarding the relative intensities of these spots were obtained. Spots intensities were expressed as percentages (% vol) of relative volumes by integrating the value (or OD) of each pixel in the spot area (vol) and dividing it with the sum of the volumes of all the spots detected in the gel.

In-gel digestion and mass spectrometry

For spot picking, preparative gels in which quantity of protein was ten times more than that in analytical gels, were prepared. After coomassie brilliant blue (CBB) staining using Coomassie Tablets, PhastGel R-350 (GE Healthcare), the protein spots of interest were excised and put into 1.5 ml eppendorf tubes. After destaining, the spots were digested with trypsin (sequencing grade, GE Healthcare) and the peptides were extracted. Prior to analysis on mass spectrometer, the peptide solutions were desalted by Zip Tip C18 (Millpore, Tokyo, Japan). The peptide solutions were then applied onto MALDI plate directly and the solution drop was allowed to air-dry. Furthermore, the matrix solution, prepared by dissolving 10 mg of α-cyano-4-hydroxycinnamic acid (CHCA, Sigma, USA) in 1 ml of 50% acetonitrile and 0.1% triflouroacetic acid in deionized water, was overlayed onto the dried drops. After the matrix solution was dried, the plate was inserted into the MALDI-TOF mass spectrometer and was subjected to peptide mass fingerprinting. All the MALDI-TOF mass spectra were acquired on AXIMA-CFR mass spectrometer. The acquired MS spectra were searched against NCBI database using the MASCOT (www.matrixscience.com) MS search engine. The search parameters were the following: type of search, peptide mass fingerprint; enzyme, trypsin; fixed modification,

carbamidomethyl (C); variable modifications, oxidation (M); mass values, monoisotopic; protein mass, unrestricted; peptide mass tolerance, 0.2 Da; peptide charge state, 1; max missed cleavages, 2.

Real-time PCR

To find the trigger genes on the neuroprotective effect of 3,5-di-O-CQA, we considered the lower concentration (10 μM) and shorter treatment time (16 h). After incubating seeded plates for 16 h, total RNA was purified using the ISOGEN kit (Nippon Gene Co. Ltd., Japan). Total RNA was quantified by measurement of the spectrophotometer at 260 nm with a UV spectrophotometer and was also measured at 280 nm to assess purity. Only RNA with a 260/280 ratio higher than 1.8 was used for real-time PCR. The template cDNA was synthesized from total RNA using the SuperScript reverse transcriptase system (Invitrogen). Briefly, RNA was denatured at 65 ℃ for 5 min and incubated with 1 μL oligo $(dT)_{12-15}$ primers and chilled at 4℃. After adding SuperScript II reverse transcriptase (200 units) the reaction mix was incubated at 42℃ for 60 min, then 10 min at 70℃ (16). For the quantification of mRNA, nested primers were designed using Primer3 input software (http://frodo.wi.mit.edu/cgi-bin/primer3/ primer3.cgi/primer3_www.cgi). Quantitative PCR reactions were performed in a MiniOpticon instrument (Bio-Rad, USA) and carried out as recommended for iQ SYBRGreen supermix (Bio-Rad). Briefly, the RT mix (2 μL) was used as template for the real-time PCR mix containing 0.5 mM forward (5' ACAATGGAGCCAAGTCGGTAG-3') and reverse (5'-GCCTACACAGTCCTTCAAGAAC-3') nested primers (2 μL each) and 2 x SYBR Green

supermix (10 μL). The amplification conditions were: 3 min at 95℃, 10 s at 95℃, 30 s at 62℃, and 30 s at 72℃ for 34 cycles. At the end of the reaction, a melting curve analysis was carried out to check for the presence of primer-dimers.

Measurement of Intracellular ATP Content.

ATP was assessed as firefly bioluminescence using the luminescence luciferase assay kit (TOYO Ink, Tokyo, Japan). To determine the increase of intracellular ATP content due to 3,5-di-O-CQA treatment SH-SY5Y cells (2 x 10^3 cells/well) were pretreated for 48 h with 3,5-di-O-CQA (10 and 20 μM) after which, cells were lysed with 100 μL of lysis buffer (Toyo ink) and placed directly into the chamber of a luminometer (Powerscan HT; Dainippon Pharmaceutical, Osaka, Japan). Light emission was recorded after the addition of 100 μL of luciferin-luciferase solution (Toyo ink). When ATP is the limiting component in a luciferase reaction, the intensity of light emitted is proportional to the concentration of ATP in the cytosolic extracts.

Animals and supplementation

Male SAMP8 and SAMR1 mice were provided by the Japan SLC company. Three mice were housed per cage with a 12-h light/dark cycle. The animals had free access to food and water. After a 7-day acclimatization to the laboratory conditions, 3 month old SAMP8 mice (*n*=20) were randomly divided into two groups: SAMP8 control group (*n*=10), 3,5-di-O-CQA-supplemented group (*n*=10), while age matched SAMR1 mice (*n*=10) served as the normal aging control. The 3,5-di-O-CQA-treated mice were orally administered with 3,5-

di-O-CQA mixed with drinking water (6.7 mg/kg · day) for 1 month using oral administration tube and syringe.

Morris water maze (MWM)

After the open field test, the MWM was selected as a method for the evaluation of the spatial learning and memory. A circular water tank (120 cm in diameter and 50 cm in height) was filled with water to a depth of 30 cm. Inside the tank, an escape platform (11 cm in diameter) was placed, with the top of 1 cm below the water surface. The platform was in the middle of the target quadrant, and its position remained fixed during the experiment. Above the tank, a white floor-to-ceiling cloth curtain was drawn around the pool, and four kinds of black cardboard (circle, triangular, rhombus and square) were hung equidistantly on the interior of the curtain serving as spatial cues. Each mouse had daily sessions of one trial for 30 consecutive days. When they succeeded, mice were allowed to stay on the platform for 30 s. When the mice failed to find the platform within 60 s, they were assisted by the experimenter and allowed to stay the platform for the same time. A probe trial was performed 24 h after the last training session. In this trial, the platform was removed from the tank and mice were allowed to swim freely for 60 s.

Statistical analysis

The escape latency of mice in the MWM training was analyzed using the two-way analysis of variance (ANOVA) with repeated measures, the factors being treatment and training day. One-way ANOVA was used to analyze group differences for the data collected in the swim

speed analysis, the MWM probe trial, and the open field test, Nissl staining and Western blotting, followed by LSD (equal variances assumed) or Tamhane's T2 (equal variances not assumed) post hoc tests. A criterion of $P<0.05$ was considered significant and the results were expressed as mean±SEM.

RESULTS

Neuroprotective effect of CQA on $A\beta_{1-42}$-induced neuronal death

In order to eliminate the possible neuroprotective effect of 3,5-di-O-CQA, we examined the cell viability when treated with 3,5-di-O-CQA and $A\beta_{1-42.}$ SH-SY5Y cells were incubated in the presence or in the absence of 2 μM $A\beta_{1-42}$ for 72h and the neuronal survival determined by MTT assays. Fig. 1 shows the MTT result of cells treated for 72 h with 10 μM $A\beta_{1-42}$, and treated with 20 μM 3,5-di-O-CQA. The cell viability of $A\beta_{1-42}$ treated cells was significantly decreased to 74.2%±7.6 compared with the non-treated group ($P<0.01$). In addition, we investigated the effect of 3,5-di-O-CQA on preventing decreased cell viability in SH-SY5Y cells induced by $A\beta_{1-42}$. Incubation with 20 μM 3,5-di-O-CQA reversed the $A\beta_{1-42}$-induced cell death and the cell viability significantly increased to 106.5%±9.8 compared to the non-treated group ($P<0.01$). Interestingly, the cell viability of 3,5-di-O-CQA treated cells, without $A\beta_{1-42}$ treatment, significantly increased to 124.6% ±9.8 compared to the non-treated group ($P<0.01$)

Effect of CQA and/or Aβ treatment on proteins expression profiles of SH-SY5Y cells

To examine the possible mechanism involved in the neuroprotective effect of 3,5-di-O-CQA on neuronal cells, we performed a proteomics analysis on $A\beta_{1-42}$-treated SH-SY5Y cells with or without 3,5-di-O-CQA treatment. Proteins were extracted from the CQA and/or Aβ treated SH-SY5Y cells, which had been treated for 72 h, and the total proteins separated by 2D-gel electrophoresis. A protein pattern of SH-SY5Y cells is shown in Fig. 2A. Approximately 2000 spots were detected in each silver-stained gel, with molecular-mass ranges of 15 to 200 kDa and a pI, that ranges from 3 to 10. Many spots from CQA and/or Aβ-treated SH-SY5Y cells were increased or decreased on each gel. As shown in Fig. 2B, $A\beta_{1-42}$ treatment caused a substantial decrease in a particular spot. However, 3,5-di-O-CQA treatment induced up-regulation of a particular spot under $A\beta_{1-42}$ treatment. The spot protein had a relative molecular mass of 40-45 kDa and an isoelectric point in the range of 7.0-7.6 (Boxed region). To identify this protein, the spot was excised and subjected to tryptic digestion and MALDI-ToF mass spectrometry analysis. The results of the database searches showed that the protein sequence most closely corresponded to that of human phosphoglycerate kinase-1 (PGK1), with matching peptides covering 67% of PGK1 (281/417 amino acids).

Effect of CQA on the mRNA expression level of PGK1

In order to determine the effect of 3,5-di-O-CQA on the mRNA expression level of PGK1, which is overexpressed by 3,5-di-O-CQA treatment on protein level, we performed the real-time PCR analysis using β-actin as a control gene. As shown in Fig. 3, the mRNA expression level of PGK1 was highly increased by 20 μM 3,5-di-O-CQA treatment on SH-

SY5Y cells. In fact, the mRNA expression level of PGK1 was significantly upregulated by 119% ±7.2 compared with the non-treated group (P<0.05).

Effects of CQA on intracellular ATP production

Based on the results of proteomics analysis and real-time PCR, 3,5-di-O-CQA induced the increase in PGK1 expression. The PGK1 protein is one of glycolytic enzymes of glycolysis. To investigate the upregulated glycolytic enzyme's effects on energy generation, the levels of ATP, which is the end product of glycolysis, were evaluated. ATP is a multifunctional nucleotide that is important as a "molecular currency" of intracellular energy transfer. In this role, ATP transports chemical energy within cells for metabolism. Intracellular ATP production level of 3,5-di-O-CQA-treated SH-SY5Y cells was measured by a luciferase reaction method. In 3,5-di-O-CQA-treated SH-SY5Y cells, luminescence was significantly upregulated by 113% ±7.2 compared to non-treated group (P<0.05) (Fig. 4).

Effect of CQA on spatial learning and memory of SAMP8 mice in MWM

We measured the time, swimming time to arrive at the platform (escape latency time), to assess the effect of 3,5-di-O-CQA on spatial learning and memory, which is AD's major symptom. We noted that the 3 month-old SAMP8 and SAMR1 mice demonstrated significant differences in motivational swimming speed between groups (data not shown). As shown in Fig. 5, all groups (SAMP8, SAMP8+CQA, SAMR1 group) improved their performance after 30 days. However, the escape latency time of SAMP8 group (n=10) was slightly decreased, compared with SAMR1 and SAMP8+CQA groups. The escape latency

time of SAMP8+CQA and SAMR1 group (n=10) was significantly decreased compared to SAMP8 group (P<0.01). There was no significant difference between SAMP8+CQA and SAMR1group on the escape latency time.

Effect of CQA on the mRNA expression level of PGK1 on SAMP8 mice brain

We investigated the mRNA expression level of PGK1 on SAMP8 mice brain to determine the effect of 3,5-O-di CQA on PGK1 expression *in vivo*. From the real-time PCR result (Fig. 6), the mRNA expression level of PGK1 was highly increased in the brain of 3,5-di-O-CQA administrated SAMP8 mice. In fact, the PGK1 mRNA expression in SAMP8+CQA group was significantly upregulated by 153% ±7.5 compared with the SAMR1 group (P<0.01). However, the PGK1 mRNA expression in SAMP8 group was slightly down-regulated compared to SAMR1 group (not significantly).

DISCUSSION

The pathogenesis of AD has been reported by several researchers who carried out post-mortem determinations. AD is induced by: (1) a cholinergic defect; (2) a glutamatergic impairment; (3) chromosome 21 impairment which produces β-APP; and (4) the endothelium impairment of blood–brain barrier (BBB) (Hagino et al., 2004). Senile dementia is a disease involving a cholinergic defect which is the direct result of the decreased glucose metabolism in the brain (Meier-Ruge et al., 1984). Since the key substrate of acetylcholine synthesis is acetylcoenzyme A, which is exclusively synthesized in the glycolytic pathway in the brain. Glucose matabolism, final products of which are

ATP and NADH, is the main fuel for all brain cells. The cholinergic defect, nerve cell death and the amyloid protein accumulation in the brain may be a secondary phenomena caused by the decline in glucose metabolism in senile dementia (Benson et al., 1983; Tucek et al., 1990).

In the presence of oxygen, glucose undergoes complete combustion to create CO_2 and H_2O. The energy produce reaction that liberates free energy, which is trapped as ATP into two consecutive processes: glycolysis and oxidative phosphorylation in mitochondria. The impairment of glycolytic metabolism of glucose in the endothelium of BBB, and the dysfunction of glycolysis pathway cause hypoglycemia in the brain which may initiate the accumulation of amyloid protein in the brain (Hagino et al., 2004).

Our results show that 3,5-di-O-CQA induced the intracellular ATP level in the human neuroblastoma clonal SH-SY5Y cells. Moreover, 3,5-di-O-CQA induced the overexpression of PGK1 mRNA on *in vivo* and *in vitro*. From these results, we suggest that the increase of intracellular ATP level is caused by overexpression of PGK1 mRNA in 3,5-di-O-CQA treatment. Therefore, it is possible that the neuroprotective effect of CQA is closely related to the energy metabolism activation, glycolysis pathway activation via the overexpression glycolytic enzyme.

CQA has a variety of physiological activities, antioxidant activity, cell differentiation, analgesic activity and neuroprotective effect. However, there has been no report that CQA induced the activation of ATP production in neuronal cells. Our study is the first report on the new function of CQA as a stimulator of ATP production. Exposure of neuronal cells to the strict circumstance, such as cell toxic and chronic hypoxia stimulates

the expression of glucose transporters and enzymes that accelerate glucose utilization to compensate for the reduced production of ATP (Gao et al., 2004). Such a response is gradually late and weak in aging cells. From our results, we suggest that CQA contribute to the homeostasis of ATP production in cells, especially on neuronal cells.

Futhermore, glycolytic enzymes have glycolytic and non-glycolytic functions. As for the non-glycolytic functions, glycolytic enzymes play an important role in: (1) apoptosis; (2) transcriptional regulation; (3) cell motility (Kim et al., 2005; Canback et al., 2002). Some glycolytic enzymes, Hexokinase, GAPD, etc, are implicated in neuronal apoptosis, and several groups have been investigating thier possible roles in age-related neurodegenerative disorders such as AD (Zheng et al., 2003; Sirover et al., 1999; Mazzola et al., 2003). These findings suggest that the activated glycolytic enzymes by CQA treatment were related to the regulation of neuronal cell apoptosis via apoptosis regulator; BAD, Bak and Bcl.

The parameter of spatial learning and memory (means of escape latency time to find the hidden platform) within 6 days in MWM in SAMP8 and SAMR1 mice was similar. However, 3,5-di-O-CQA, 6.7 mg/kg · day, were shown to have decreased escape latency time compared to the vehicle-administrated SAMP8 mice for 10-30 days. This dose of 3,5-di-O-CQA also caused an increase in PGK1 mRNA expression in SAMP8 mice brains (Fig. 6). From these results, 3,5-di-O-CQA induced the overexpression of PGK1 mRNA level both *in vitro* and *in vivo*.

Particularly, PGK1 is an important glycolytic enzyme because this enzyme that induces the production of 2 molecules of ATP in glycolysis pathway. Furthermore, PGK1

is not a rate limiting enzyme which is Hexokinase, Phosphofructokinase, Pyruvate kinase, in the glycolysis pathway (Theresa et al., 1982). Therefore, the activation of PGK1 enzyme is not affected by the feedback inhibition on ATP production. We suggest that 3,5-di-O-CQA can induced the activation of ATP production without feedback inhibition through the activation of PGK1.

CONCLUSION

In conclusion, we found that 3,5-di CQA has a neuroprotective effect on $A\beta_{1-42}$ treated SH-SY5Y cells. The mRNA expression of glycolytic enzyme (PGK1) and the intracellular ATP level were increased in 3,5-di-O-CQA-treated SH-SY5Y cells. We also found that 3,5-di-O-CQA administration induced the improvement of spatial learning and memory on SAMP8 mice, and the overexpression of PGK1 mRNA level. These findings suggest that 3,5-di-O-CQA has a neuroprotective effect through the induction of PGK1 expression and ATP production activation.

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Fig. 1

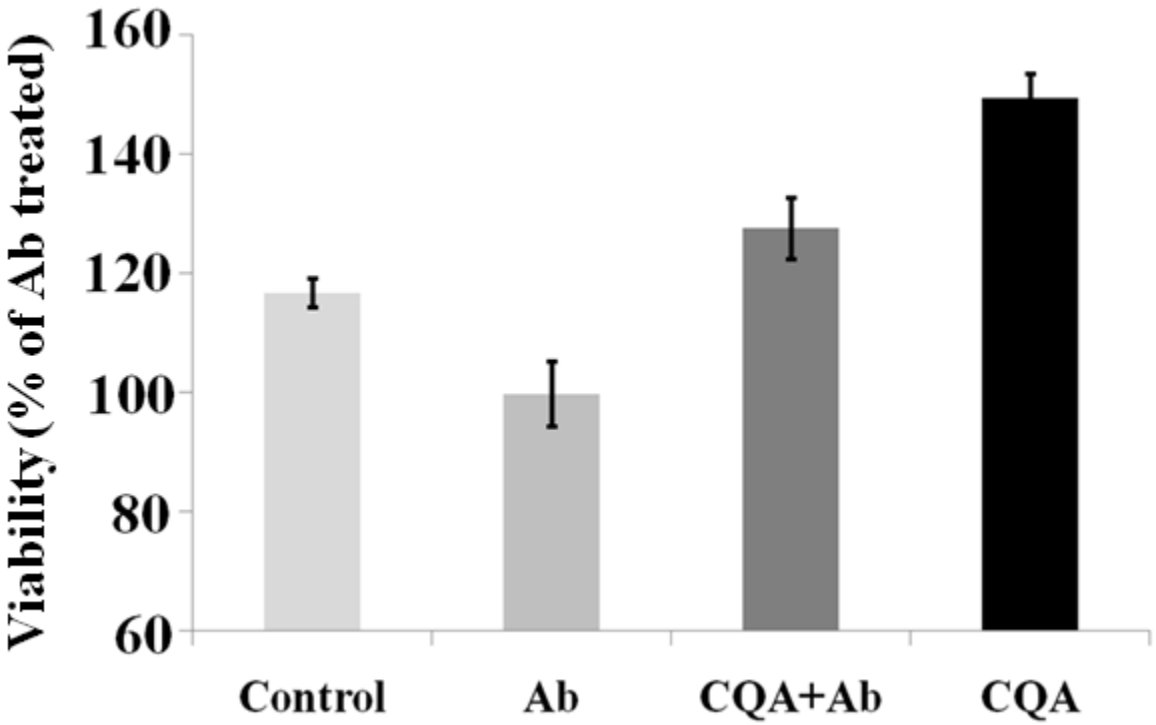
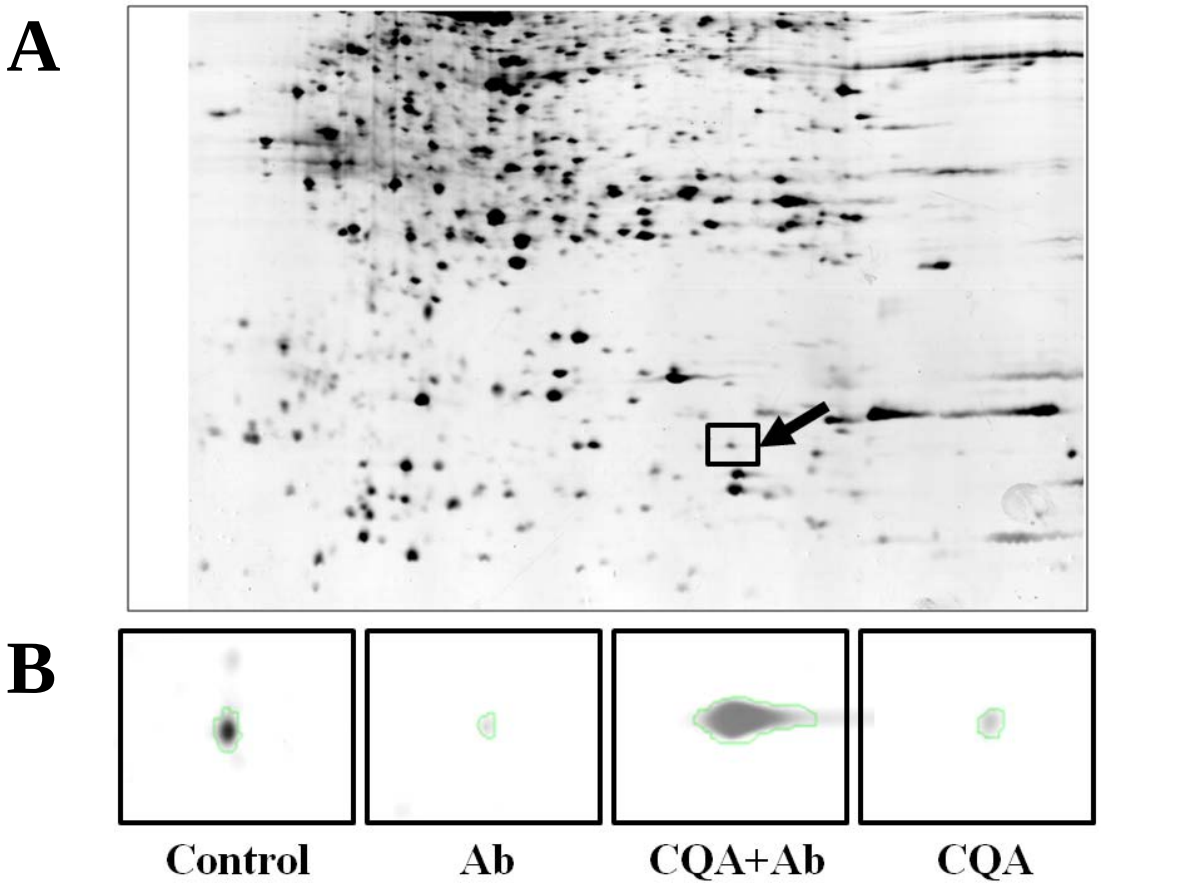

Viability (% of Ab treated)
160
140
120
100
80
60
Control
Ab
CQA+Ab
CQA

Fig. 2


A
B
Control
Ab
CQA+Ab
CQA

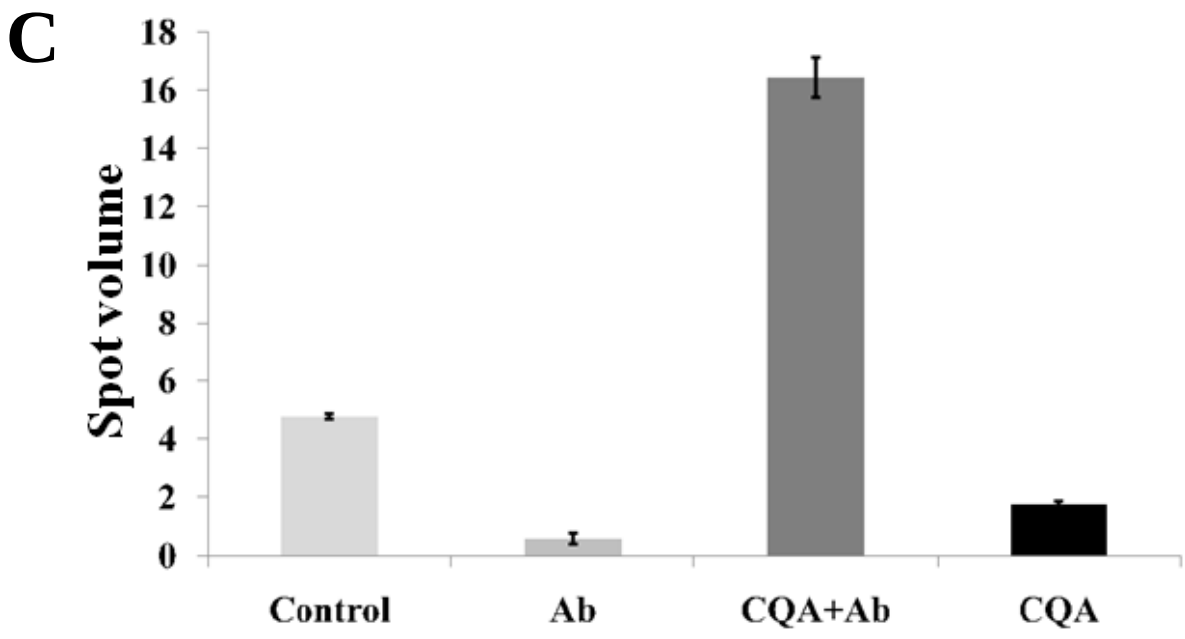

C
Spot volume
18
16
14
12
10
8
6
4
2
0
Control
Ab
CQA+Ab
CQA

Fig. 3

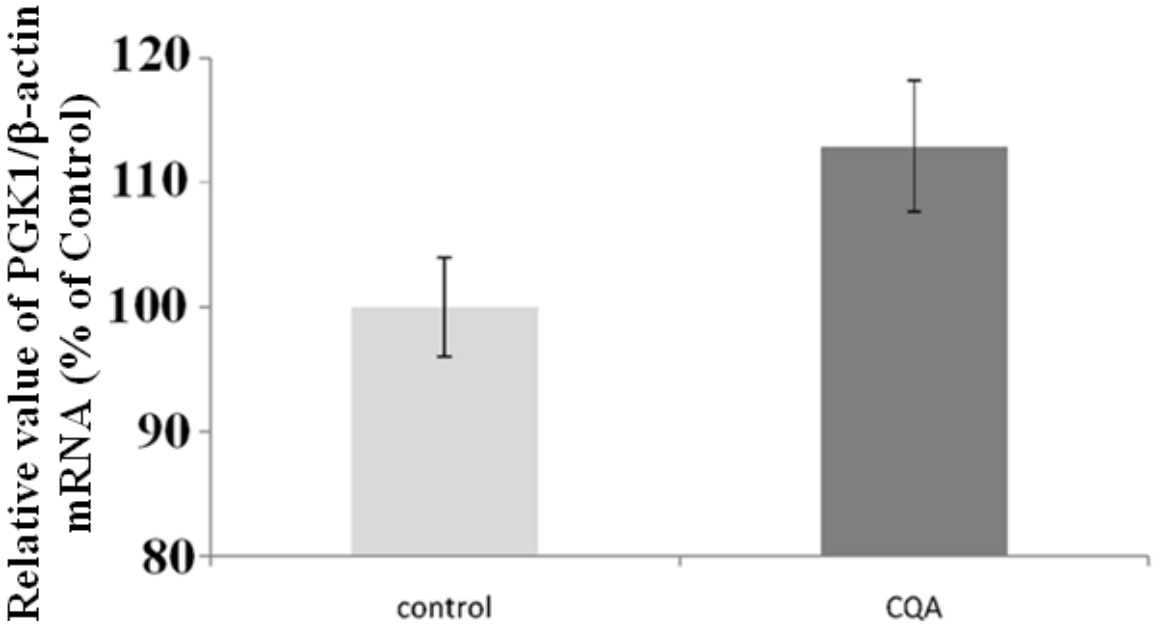

Relative value of PGK1/β-actin mRNA (% of Control)
120
110
100
90
80
control
CQA

Fig. 4

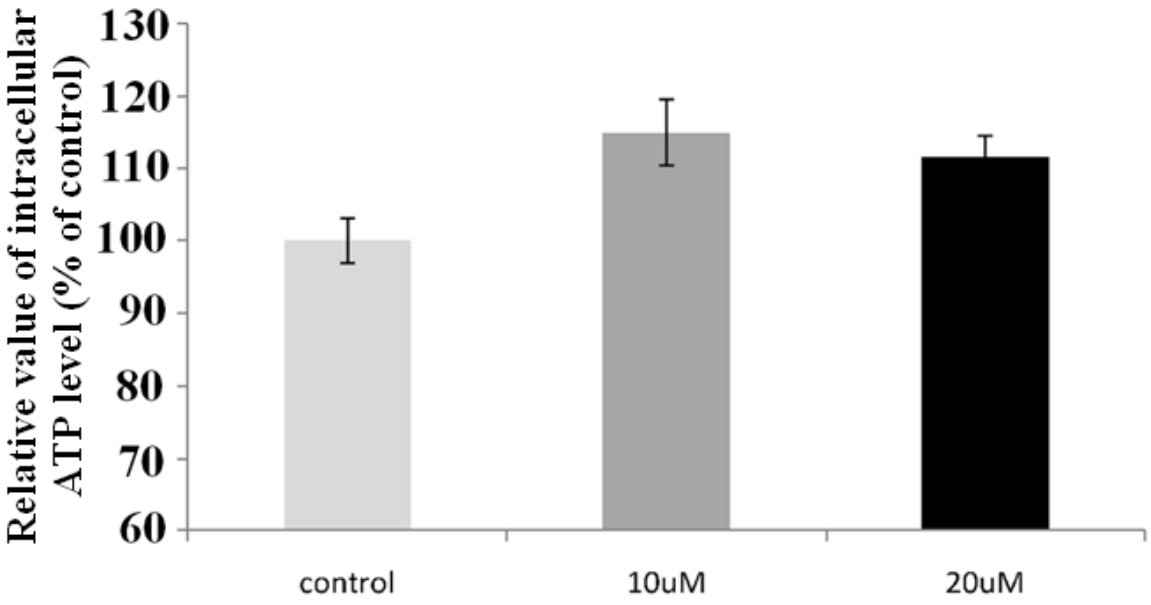

Relative value of intracellular ATP level (% of control)
130
120
110
100
90
80
70
60
control
10uM
20uM

Fig. 5

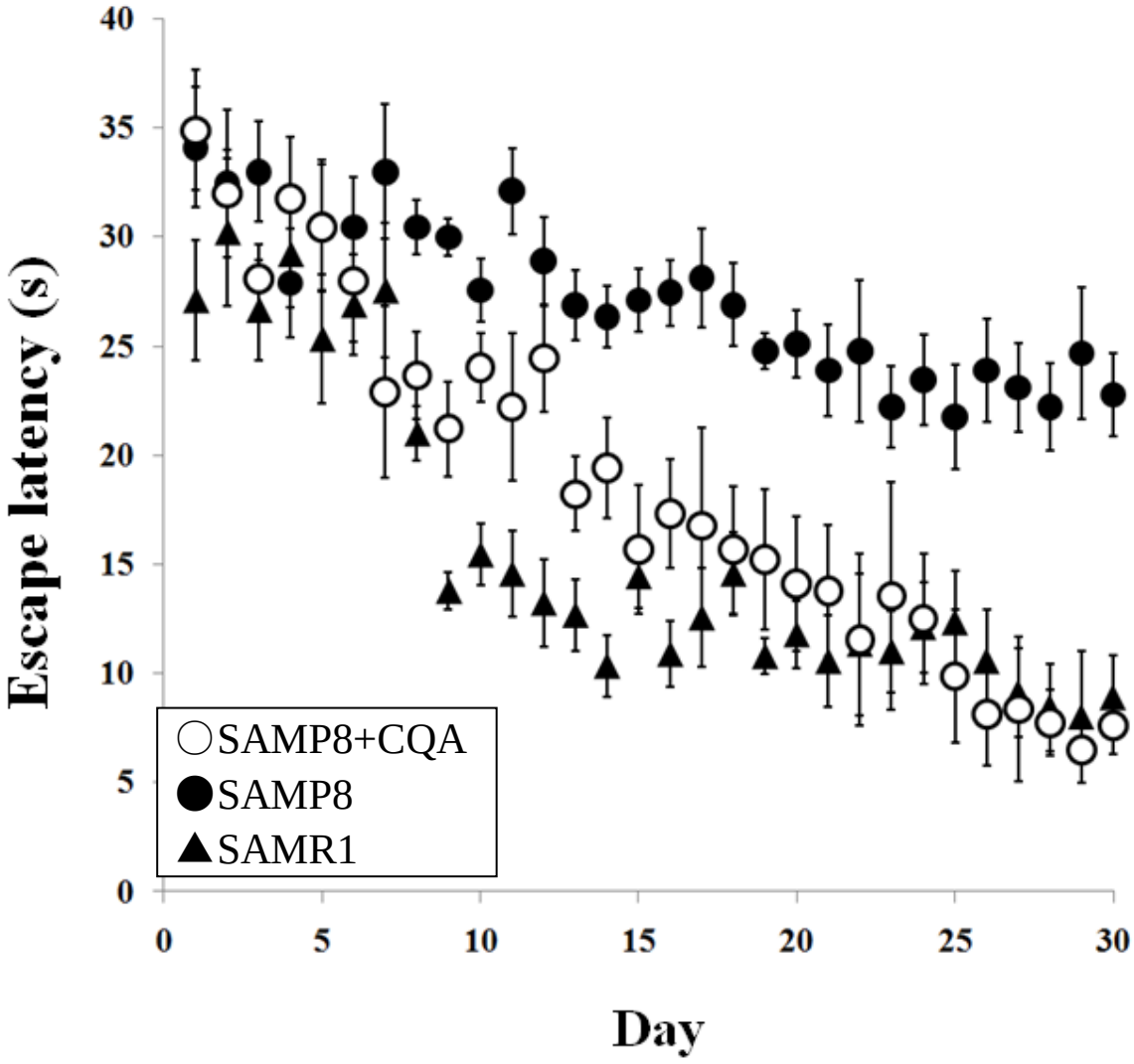

Escape latency (s)
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
Day
SAMP8+CQA
SAMP8
SAMR1

Fig. 6

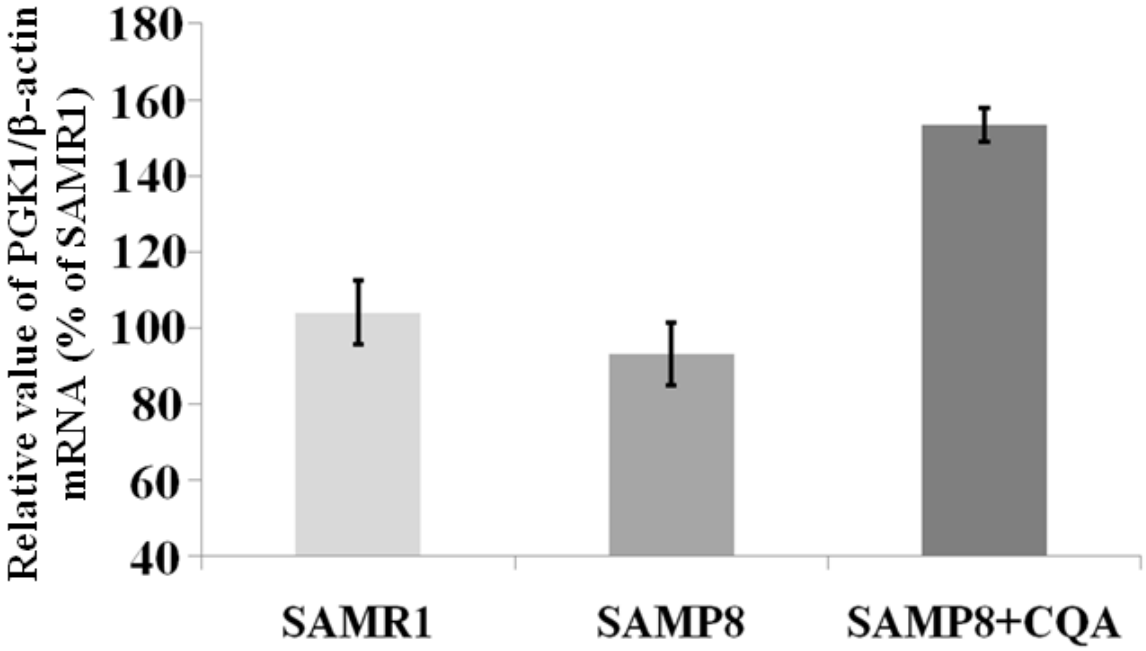

Relative value of PGK1/β-actin mRNA (% of SAMR1)
180
160
140
120
100
80
60
40
SAMR1
SAMP8
SAMP8+CQA

Fig. 1 Effect of 3,5-di-O-CQA on the $A\beta_{1-42}$ treated SH-SY5Y cells viability. SH-SY5Y cells were treated with 20 μM 3,5-di-O-CQA (CQA) or 2 μM $A\beta_{1-42}$ (Aβ) for 72 h. Each bar represents the mean ± SD (n = 4). *P<0.05

Fig. 2 Two-dimensional gel electrophoresis of SH-SY5Y cells (A), the magnified images of the boxed regions (B) and spot volume (C). SH-SY5Y cells were treated with 20 μM 3,5-di-O-CQA or exposed to 2 μM $A\beta_{1-42}$ for 72 h,. The 2-DE gel was stained with coomassie brilliant blue. Spot volume was measured by ImageMaster 2D Platinum software. These spots were identified as PGK1 by MALDI-TOF mass spectrometry. Each bar represents the mean ± SD (n = 3). **P<0.01 (vs control).

Fig. 3 Effect of 3,5-di-O-CQA on the expressions of PGK1 mRNAs by SH-SY5Y cells. β-actin was used as a housekeeping gene. The mRNA expression of PGK1 was normalized by β-actin mRNA expression. SH-SY5Y cells were treated with 10 μM 3,5-di-O-CQA for 16 h. Each bar represents the mean ± SD (n = 4). *P<0.05

Fig. 4 Effect of 3,5-di-O-CQA on the intracellular ATP production of SH-SY5Y cells. SH-SY5Y cells were treated with 10 and 20 μM 3,5-di-O-CQA for 48 h. Intracellular ATP production was increased by 3,5-di-O-CQA treatment on SH-SY5Y cells. Each bar represents the mean ± SD (n = 10). **P<0.01 vs control

Fig. 5 Effect of 3,5-di-O-CQA on the spatial learning and memory of SAMP8 mice in MWM. The average time to reach the hidden platform (latency in seconds) is plotted for each training day. SAMP8 mice were administrated with 3,5-di-O-CQA (6.7 mg/kg・day) for 30 days. Each bar represents the mean ± SD (n = 8). *P<0.05 vs SAMP8 group.

Fig. 6 Effect of 3,5-di-O-CQA on the expressions of PGK1 mRNAs by SAMR1 and SAMP8 mice brains. β-actin was used as a housekeeping gene. The mRNA expression of PGK1 was normalized by β-actin mRNA expression. SAMP8 mice were administrated with 3,5-di-O-CQA (6.7 mg/kg・day) for 30 days. Each bar represents the mean ± SD (n = 8). *P<0.05