3,4,5-tri-*O*-caffeoylquinic acid inhibits amyloid β -mediated cellular toxicity on SH-SY5Y cells through the upregulation of PGAM1 and G3PDH

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Abstract Caffeoylquinic acid (CQA) is one of the phenylpropanoids found in a variety of natural resources and foods, such as sweet potatoes, propolis, and coffee. Previously, we reported that 3,5-di-*O*-caffeoylquinic acid (3,5-di-CQA) has a neuroprotective effect against amyloid- β ($A\beta$)-induced cell death through the overexpression of glycolytic enzyme. Additionally, 3,5-di-CQA administration induced the improvement of spatial learning and memory on senescence accelerated-prone mice (SAMP8). The aim of this study was to investigate whether 3,4,5-tri-*O*-caffeoylquinic acid (3,4,5-tri-CQA), isolated from propolis, shows a neuroprotective effect against $A\beta$ -induced cell death on human neuroblastoma SH-SY5Y cells. To clarify the possible mechanism, we performed proteomics and real-time RT-PCR as well as a measurement of the intracellular

adenosine triphosphate (ATP) level. These results showed that 3,4,5-tri-CQA attenuated the cytotoxicity and prevented $A\beta$ -mediated apoptosis. Glycolytic enzymes, phosphoglycerate mutase 1 (PGAM1) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were overexpressed in co-treated cells with both 3,4,5-tri-CQA and $A\beta$. The mRNA expression of PGAM1, G3PDH, and phosphoglycerate kinase 1 (PGK1), and intracellular ATP level were also increased in 3,4,5-tri-CQA treated cells. Taken together the findings in our study suggests that 3,4,5-tri-CQA shows a neuroprotective effect against $A\beta$ -induced cell death through the upregulation of glycolytic enzyme mRNA as well as ATP production activation.

Keywords 3,4,5-tri-*O*-caffeoylquinic acid · SH-SY5Y cells · G3PDH · PGAM1 · Glycolytic enzyme · ATP

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Introduction

Alzheimer's disease (AD) is neuropathologically characterized by the deposition of amyloid- β ($A\beta$) plaques intracellular neurofibrillary tangles and loss of neurons in the brain. $A\beta$ -induced neurotoxicity implicates oxidative stress that is associated with an increase in lipid peroxidation, protein oxidation, alteration of antioxidant systems, and DNA damage as described in AD brains (Miranda et al. 2000;

Qi et al. 2005; Ramassamy et al. 1999). Also, the residue methionine 35 in the $A\beta_{1-42}$ peptide plays a critical role in oxidative stress and its neurotoxic properties (Butterfield and Boyd-Kimball 2005). Additionally, mitochondrial dysfunction is suggested to be involved in $A\beta$ -induced oxidative cell death (Atamna and Frey 2007; Cardoso et al. 2001; Ferrer 2009; Yang et al. 2008). Furthermore, various types of compounds, such as acteoside, ginkgolide, and galantamine, have been shown to inhibit $A\beta$ -induced neurotoxicity (Liu et al. 2009; Matharu et al. 2009; Shi et al. 2009; Wang et al. 2009).

Caffeoylquinic acid (CQA) is one of the phenylpropanoids found in coffee beans, sweet potatoes, propolis, and other plants (Clifford et al. 2007; Farah et al. 2005; Merfort 1992). CQA derivatives have a variety of bioactivities, such as antioxidant, antibacterial, anticancer, antihistamic as well as other biological effects (Kimura et al. 1985; Kurata et al. 2007; Matsui et al. 2004; Mishima et al. 2005; Yoshimoto et al. 2002). In our previous study, we demonstrated that 3,5-di-*O*-caffeoylquinic acid (3,5-di-CQA) inhibited $A\beta_{1-42}$ -induced cellular toxicity on human neuroblastoma SH-SY5Y cells and increased the mRNA expression level of glycolytic enzyme, phosphoglycerate kinase 1 (PGK1) as well as the intracellular adenosine triphosphate (ATP) level (Han et al. 2010). Moreover, we also found that 3,5-di-CQA administration induced the improvement of spatial learning and memory on senescence accelerated-prone mice (SAMP8), and resulted in the overexpression of the PGK1 mRNA level. However, it is unclear whether any type of caffeoylquinic acid possesses a neuroprotective effect as well as that of 3,5-di-CQA. For the diversity of the CQA content in the food resources, it is important to clarify the neuroprotective activity of individual CQAs; therefore, we focused on 3,4,5-tri-*O*-caffeoylquinic acid (3,4,5-tri-CQA; Fig. 1). Previously, we isolated 3,5-di-CQA from *Cuscuta pentagona*, parasitic plant. However, 3,4,5-tri-CQA are rare in this plant. In this study, we used Brazilian propolis as an alternative source of CQA. Propolis is a resinous substance collected by honeybees from exudates and buds of plants and mixed with secreted beeswax. Propolis has been used in folk medicine from ancient times. In modern times, it has been found to have a wide range of biological activities, such as antibacterial, anti-inflammatory, antioxidative, and anticancer. Propolis

Fig. 1 Structure of 3,4,5-tri-*O*-caffeoylquinic acid

contains various constituents, including flavonoids, phenolic acids, cinnamic acid derivatives. Additionally, it has been known that it abundantly contains caffeoylquinic acid derivatives.

In this study, we isolated 3,4,5-tri-*O*-caffeoylquinic acid from propolis and examine the neuroprotective effect against $A\beta$ -mediated cytotoxicity in SH-SY5Y cells. Furthermore, to investigate the mechanism of the neuroprotective effect of 3,4,5-tri-CQA, we comprehensively analyzed the change of protein expression level by (2D)-polyacrylamide-gel electrophoresis (PAGE), matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry analysis.

Materials and methods

Purification of 3,4,5-tri-CQA

Brazilian propolis (50 g) was extracted with MeOH (450 mL) and evaporated to dryness *in vacuo* at 30 °C. The MeOH extract (29.6 g) was partitioned with EtOAc (1 L $\times$ 3) and H₂O (1 L). The EtOAc-soluble portion (9 g of 22.9 g) was divided into six fractions using silica gel column chromatography (4.4 $\times$ 35 cm, CHCl₃/MeOH, 95:5 to 0:100). A fraction (384 mg) eluted with CHCl₃/MeOH (50:50) was subjected to ODS column chromatography (1.1 $\times$ 35 cm, MeOH/H₂O, 30:70 to 100:0). A fraction (132 mg) eluted with MeOH/H₂O (100:0) was applied to a C₁₈ Sep-Pak cartridge (Waters, MeOH/H₂O, 70:30 to 100:0) to give 3,4,5-tri-CQA (71.5 mg). For each assay, 3,4,5-tri-CQA was dissolved in EtOH and added to the cell culture medium.

Cell culture

The human dopaminergic neuroblastoma SH-SY5Y cell line was obtained from American Type Culture Collection (ATCC). Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with 50% F-12 nutrient mixture (F-12, GIBCO), 15% fetal bovine serum (FBS), and 1% penicillin (5,000 U/mL)—streptomycin (5,000 µg/mL) in a 100 mm fibronectin coated dish (BD, Biocoat). The cells were grown at 37 °C in 5% CO₂. To culture for the subsequent extraction of protein and total RNA, cells were seeded onto Petri dishes at a density of 2×10^6 cells per dish, and 3,4,5-tri-CQA was added at a final concentration of 20 µM.

MTT assay

SH-SY5Y cells were cultured in 100 µL of test medium at a density of 2×10^4 cells/well in a fibronectin coated 96-well micro-plate (BD, Biocoat). After a 24 h incubation period at 37 °C (5% CO₂), cells were treated with 3,4,5-tri-CQA dissolved in 100 µL of OPTI-MEM (GIBCO) and were exposed to 10 µM of Aβ_{1–42} (Funakoshi, Tokyo) for 72 h. Subsequently, the culture medium was changed to 100 µL of OPTI-MEM without CQAs or Aβ. 10 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; DOJINDO) dissolved in PBS (–) at 5 mg/mL was added. After overnight incubation, the colored formazan was dissolved in 100 µL of 10% SDS. The absorbance at 570 nm was determined using a multi-detection micro plate reader (Powerscan®, HT, Dainippon Pharmaceutical, Japan), and the viability of SH-SY5Y cells was presented as percentage of that of the control.

Proteome analysis

Two-dimensional gel electrophoresis (2-DE)

2-DE was performed as described by Isoda et al. (2006) with modification. Samples containing 30 µg for analytical gel, or 300 µg for preparative gel of protein, were separated by isoelectric focusing (IEF) and then sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using Ettan IPGphor II and Ettan DALTsix (GE Healthcare, Uppsala,

Sweden). For IEF, samples were added to rehydration solution containing 8 M urea, 2% CHAPS, 0.5% IPG buffer, 0.002% bromophenol blue, and 0.28%(w/v) dithiothreitol (DTT), and were applied to 24-cm 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 °C for 12 h, and isoelectric focusing of proteins was carried out at 500 V for 1 h, 1,000 V for 1 h, 10,000 V for 3 h, and 10,000 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 a Plus One Silver Staining Kit (GE Healthcare) was performed according to the manufacturer's instructions. Briefly, analytical gels after 2-DE were fixed in fixative solution (40% ethanol and 10% acetic acid) overnight and were sensitized in solution containing 30% ethanol, 25% (w/v) glutardialdehyde, 5% (w/v) sodium thiosulphate, and 6.8% sodium acetate. The gels were stained in solution with 2.5% (w/v) silver nitrate solution and 0.04% formaldehyde (37% w/v) followed by development in a solution of 2.5% (W/V) sodium carbonate and 0.08% formaldehyde. Staining was stopped by a stop solution (1.46% EDTA-Na₂·2H₂O). After washing with distilled water, the gels were subjected to image analysis using Image-Master 2D Platinum software (ver. 4.9; GE Healthcare). After spot detecting (matching), the spots of interest were manually selected, and data regarding the relative intensities of these spots were obtained. Spot 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 by the sum of volumes of all spots detected in the gel.

In-gel digestion and mass spectrometry

Spot picking was performed on preparative gels in which quantity of protein was ten times higher than that in analytical gels. 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 a mass spectrometer, the peptide solutions were desalted by Zip Tip C₁₈ (Millipore, Tokyo, Japan). The peptide solutions were then applied onto a MALDI plate directly, and the solution drop was allowed to air-dry. The matrix solution was prepared by dissolving 10 mg of α -cyano-4-hydroxycinnamic acid (CHCA, Sigma, USA) in 1 mL of 50% acetonitrile, and 0.1% trifluoroacetic acid in deionized water was overlaid onto the dried drops. After the matrix solution was dried, the plate was inserted into a MALDI-TOF mass spectrometer and subjected to peptide mass fingerprinting. All MALDI-TOF mass spectra were acquired on AXIMA-CFR mass spectrometer. The acquired MS spectra were searched against the National Center for Biotechnology Information (NCBI) database using the MASCOT (www.matrixscience.com) MS search engine, and the search parameters included 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; and max missed cleavages, 2. Probability-based MOWSE scores were estimated by comparison of search results against estimated random match population. MOWSE scores greater than 60 were considered to be significant ($p < 0.05$).

Quantitative real-time RT-PCR

The mRNA expression of G3PDH, PGAM1, and PGK1 was determined by real-time RT-PCR using β -actin as an internal control. Total RNA was extracted by ISOGEN kit (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions. Reverse transcription reactions were carried out with the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). Briefly, RNA was denatured at 65 °C for 5 min, incubated with 1 mL oligo (dT)_{12–15} primer and chilled at 4 °C. After adding SuperScript III reverse transcriptase (200 units) the reaction mixture was incubated at 42 °C for 60 min and then for 10 min at 70 °C. 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](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/)). Quantitative PCR reactions were performed in a MiniOpticon instrument (Bio-Rad, USA) and were carried out as recommended for iQ SYBR Green supermix (Bio-Rad). The primer sequences were as follows: β -actin Forward 5'-CTGTGGCATCCACGAACTACC-3'; β -actin Reverse 5'-GCAGTGATCTCCTTCTGCATCC-3'; G3PDH Forward 5'-CACCAGGTGGTCTCCTCTGAC-3'; G3PDH Reverse 5'-ATGAGGTCCACCACCCTGTTG-3'; PGAM1 Forward 5'-ATGCTAAGCCATGACCAGTGAG-3'; PGAM1 Reverse 5'-ATCACCACGCAGGTACATTCG-3'; PGK1 Forward 5'-CACTCGGGCTAAGCAGATTGTG-3'; PGK1 Reverse 5'-CCACCACTATGATGGTGTATGC-3'. The amplification conditions included the following: 3 min at 95 °C, 10 s at 95 °C, 30 s at 60 °C, and 15 s at 72 °C for 35 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 by firefly bioluminescence using the luminescence luciferase assay kit (TOYO Ink, Tokyo, Japan). SH-SY5Y cells were inoculated onto 96-well microplates as mentioned in the MTT assay. The cells were incubated and allowed to attach for 24 h and were then treated with different concentrations of CQAs. After 48 h, the medium was changed to 100 μ L of OPTI-MEM without CQAs. ATP content was measured according to the manufacturer's protocol.

DPPH radical scavenging assay

To measure antioxidant activity of 3,4,5-tri-CQA, a 1,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was carried out. 190 μ L of a reaction mixture containing 0.2 mM DPPH radical solution, 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (pH 6.1), and 10 μ L of 3,4,5-tri-CQA solution were added to each well of 96-well plate. The solution was mixed and incubated for 10 min at room temperature and the absorbance at 520 nm was measured. The DPPH radical-scavenging activity was determined as the percentage decrease in the absorbance of control.

Statistical analysis

All values in the figures are expressed as mean $\pm$ SD of three independent experiments. Statistical differences

between groups were determined using a Student's *t*-test, with $p < 0.05$ as the level of significance.

Results

Purification of 3,4,5-tri-*O*-caffeoylquinic acid

Brazilian propolis (50 g) was extracted with MeOH. The MeOH extracts were partitioned with EtOAc and H₂O, and the EtOAc-soluble portion (9 g) was subjected to silica gel column chromatography, separated through an ODS column chromatography and a C₁₈ Sep-Pak cartridge to give 3,4,5-tri-CQA.

Neuroprotective effect of 3,4,5-tri-CQA

The neuroprotective effect of 3,4,5-tri-CQA was investigated by an MTT assay (Fig. 2). A β (10 μ M) induced a significant decrease in cell viability (55.7%). The co-treatment of SH-SY5Y cells with 3,4,5-tri-CQA resulted in a dose-dependent protection against A β toxicity at concentrations of 5, 10, and 20 μ M, and the most significant protection was found at a concentration of 20 μ M. On the other hand, the treatment of 3,4,5-tri-CQA alone (5, 10, and 20 μ M) resulted in increase in cell viability of 21, 23, 38%, respectively. We hypothesized that 3,4,5-tri-CQA induced cell proliferation and performed GUAVA viacount assay. However, the number of SH-SY5Y cells treated with 3,4,5-tri-CQA alone was not increased (data not shown). Therefore, we concluded that 3,4,5-tri-CQA had no effect on cell number.

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

To examine the possible mechanism involved in the neuroprotective effect of 3,4,5-tri-CQA on neuronal cells, we performed a proteomics analysis on A β _{1–42}-treated SH-SY5Y cells with or without 3,4,5-tri-CQA. Proteins were extracted from SH-SY5Y cells, which had been treated with CQA and/or A β for 72 h, and the total proteins were separated by 2D-gel electrophoresis (Fig. 3a). Stained gels were analyzed with image analysis software. Approximately 2,000 spots were detected in each silver-stained gel, with molecular-masses ranging from 15 to 200 kDa, and a pI that ranged from 3 to 10. A β _{1–42} treatment caused

Fig. 2 Effect of 3,4,5-tri-CQA on the A β _{1–42} treated SH-SY5Y cells viability. SH-SY5Y cells were treated with 3,4,5-tri-CQA or 10 μ M A β _{1–42} (A β) for 72 h. Each bar represents the mean $\pm$ SD (n = 10). ** $p < 0.01$ versus control, ### $p < 0.01$ versus the cells treated only A β

a substantial decrease in several spots. Among the spots in co-treatment with A β _{1–42} and CQA, we focused on two particular spots upregulated more than two fold compared with only A β treatment. In spot two proteins had a relative molecular mass of 20–30 kDa and an isoelectric point (pI) in the range of 6.0–7.0 (Fig. 3b). To identify these proteins, the spots were excised and subjected to tryptic digestion and MALDI-TOF mass spectrometry analysis (Table 1). The results of database searches showed that the proteins sequences most closely corresponded to those of human glyceraldehyde-3-phosphate dehydrogenase (G3PDH; spot 1) and human phosphoglycerate mutase 1 (PGAM1; spot 2), with matching peptides covering 55% of human G3PDH (184/335 amino acids) and 90% of human PGAM1 (229/254 amino acids).

Effect of 3,4,5-tri-CQA on the mRNA expression levels of PGAM1, PGK1, and G3PDH

Our previous study showed that 3,5-di-CQA induced overexpression of a glycolytic enzyme, phosphoglycerate kinase 1 (PGK1) (Han et al. 2010). We evaluated the mRNA expression levels of not only G3PDH and PGAM1 but also PGK1 by quantitative real-time RT-PCR using β -actin as a control gene (Fig. 4a, b, c). The mRNA expression levels of G3PDH, PGAM1,

Fig. 3 Two-dimensional gel electrophoresis of SH-SY5Y cells (a), the magnified images of box regions (b). SH-SY5Y cells were treated with 20 μ M 3,4,5-tri-CQA or exposed to 10 μ M A β _{1–42} for 72 h. The 2-DE gel was stained with coomassie brilliant blue. Spot value was measured by ImageMaster 2D Platinum software

Table 1 Identification result from the upregulated protein spots in human SH-SY5Y neuroblastoma cell treated with both A β and CQA

Spot No.	Protein name	Accession No.	Symbol	MW (Exp./The.)	pI ^a (Exp./The.)	MOWSE Scores	Intensity ^b
Spot 1	Glyceraldehyde-3-phosphate dehydrogenase	gi120649	G3PDH	28.3/36.2	6.0/8.57	82	2.1
Spot 2	Phosphoglycerate mutase 1	gi4504753	PGAM1	24.0/28.9	6.7/6.67	235	2.3

^a pI (isoelectric point)

^b Spot volume of A β and CQA co-treatment was represented by intensity against A β alone-treatment

and PGK1 proteins were increased by 10 μ M 3,4,5-tri-CQA treatment of SH-SY5Y cells. In fact, the mRNA expression levels of G3PDH, PGAM1, and PGK1 increased by 21, 35, and 29%, respectively.

Effect of 3,4,5-tri-CQA on intracellular ATP production

Based on the results of proteomics analysis and RT-PCR, 3,4,5-tri-CQA induced an increase of glycolytic enzyme expression. To investigate the upregulated glycolytic enzyme's effects on energy generation, levels of ATP, which is the end product of glycolysis, were evaluated (Fig. 5). ATP is a multifunctional nucleotide that is most important as a

molecular currency of intracellular energy transfer. In this role, ATP transports chemical energy within cells for metabolism. Intracellular ATP accumulation of 3,4,5-tri-CQA-treated SH-SY5Y cells was measured by a luciferase reaction method. In 3,4,5-tri-CQA-treated cells (5 and 10 μ M), luminescence was about 25% increased compared to control cells.

Antioxidant activity of 3,4,5-tri-CQA

We measured the radical-scavenging activity of 3,4,5-tri-CQA by the DPPH radical-scavenging assay. As shown in Fig. 6, 3,4,5-tri-CQA exhibited a strong radical-scavenging activity in concentration dependent manner.

Fig. 4 Effect of 3,4,5-tri-CQA on the expressions of G3PDH (a), PGAM1 (b), and PGK1 (c) mRNAs by SH-SY5Y cells. β -actin was used as a housekeeping gene. The mRNA expression of PGAM1 and PGK1 was normalized by β -actin mRNA expression. SH-SY5Y cells were treated with 10 μ M 3,4,5-tri-CQA for 8 and 16 h. Each bar represents the mean $\pm$ SD ($n = 4$). * $0.01 < p < 0.05$ versus control cell, ** $p < 0.01$ versus control cell

Discussion

There is abundant evidence suggesting that $A\beta$ contributes to the pathogenesis of AD. It is now well known that $A\beta$ is neurotoxic to neuronal cells via an oxidative stress-dependent apoptotic process (Miranda et al. 2000; Qi et al. 2005; Ramassamy

Fig. 5 Effect of 3,4,5-tri-CQA on the intracellular ATP production of SH-SY5Y cells. SH-SY5Y cells were treated with 1, 5, and 10 μ M 3,4,5-tri-CQA for 48 h. Intracellular ATP production was increased by 3,4,5-tri-CQA treatment on SH-SY5Y cells. Each bar represents the mean $\pm$ SD ($n = 10$). * $0.01 < p < 0.05$ versus control cell, ** $p < 0.01$ versus control cell

Fig. 6 DPPH radical scavenging activity of 3,4,5-tri-CQA. Each bar represents the mean $\pm$ SD ($n = 6$) of the inhibition of DPPH radical (% inhibition)

et al. 1999). $A\beta$ aggregates the neuronal cell membrane and induces oxidative stress (Butterfield and Boyd-Kimball 2005; Qi et al. 2005). Intracellular ATP depletion causes cell death in neuronal cells, which leads to neurodegenerative diseases, such as AD (Atamna and Frey 2007; Cardoso et al. 2001; Ferrer 2009; Yang et al. 2008); therefore, blockage of $A\beta$ -induced neurotoxicity may help to prevent the occurrence or progression of AD. In our previous study, we indicated that 3,5-di-CQA has neuroprotective activity against $A\beta$ toxicity through activation of glycolytic pathway and ATP production (Han et al. 2010). In this study, we evaluated the neuroprotective effect of 3,4,5-tri-CQA, isolated from propolis, against $A\beta$ -induced cytotoxicity and examined its mechanism.

We hypothesized that cell proliferation by CQA was involved in the mechanism of the neuroprotective

effect because treatment with only 3,4,5-tri-CQA (5, 10, and 20 μ M) resulted in an increase in cell viability by 21, 23, and 38%, respectively (Fig. 2). However, 3,4,5-tri-CQA had no cell proliferative effect according to a cell count assay by GUAVA PCA (data not shown). Furthermore, there was no effect on cell cycle in cells treated with 3,4,5-tri-CQA (data not shown). These results indicate that 3,4,5-tri-CQA activates a metabolism enzyme and respiratory chain activity because MTT is reduced by active mitochondria in living cells (Mosmann 1983). In addition, 3,4,5-tri-CQA increases the expression level of glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and phosphoglycerate mutase 1 (PGAM1), as well as intracellular ATP level (Figs. 3, 4, 5). Consequently, we suggest that 3,4,5-tri-CQA significantly attenuated $A\beta$ -induced neurotoxicity through activation on energy metabolism, especially upregulation of glycolytic enzymes G3PDH and PGAM1 as well as activation of ATP production.

Glycolysis is a cellular energy metabolism pathway, and glycolytic enzymes have glycolytic and non-glycolytic functions. As for the non-glycolytic functions, glycolytic enzymes play important roles in apoptosis, cell motility, and modulation of cell senescence (Canback et al. 2002; Kim and Dang 2005; Kondoh et al. 2005). It was shown that some glycolytic enzymes, such as hexokinase and GAPD, are implicated in neuronal apoptosis, and several research groups have been investigating their possible roles in age-related neurodegenerative disease, AD (Mazzolaa and Sirover 2003; Sirover 1999; Zheng et al. 2003). These findings suggest that the activated glycolytic enzymes by CQA treatment were related to the regulation of neuronal cell apoptosis regulators, BAD, Bax, and Bcl.

G3PDH is the enzyme related to the reaction of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate, while PGAM modulates the reaction of 3-phosphoglycerate to 2-phosphoglycerate in glycolysis. This glycolytic enzyme induces the production of two molecules of ATP in the glycolysis pathway. Recent studies show that several glycolytic enzymes (aldolase A, aldolase C, triose phosphate isomerase, phosphoglycerate kinase (PGK), and PGAM) are oxidatively damaged in advanced AD stages (Castegna et al. 2003; Korolainen et al. 2005; Korolainen et al. 2006; Reed et al. 2008; Sultana et al. 2006). It has also been suggested that PGAM1 in the rat brain was oxidized by $A\beta_{1-42}$ -injection (Boyd-Kimball et al.

2005). Neuronal cell death is caused by ATP depletion following the glycolysis enzyme oxidation and energy metabolism inhibition. In this study, we showed that CQA has neuroprotective effects against $A\beta$ -mediated apoptosis through the upregulation of glycolysis enzyme and activation of ATP production. These findings indicate the possibility of CQA to be a potential therapeutic tool for AD through the activation of energy metabolism.

Recently, various types of compounds have been shown to inhibit $A\beta$ -induced neurotoxicity. Most of their mechanism are due to their antioxidant effect, because $A\beta$ induced apoptosis mainly through oxidative stress. As shown in Fig. 6, 3,4,5-tri-CQA also exhibit a strong radical-scavenging activity concentration dependent manner. It is thought that the antioxidant activity of 3,4,5-tri-CQA contribute to its neuroprotective effect. However, in the present study, we found that 3,4,5-tri-CQA has a neuroprotective effect against $A\beta$ -mediated cellular toxicity through the accelerating activity on energy metabolism in neuronal cells. This is the first report that the antioxidant substances such as CQA have the accelerating activity on energy metabolism in neuronal cell. The relation between the antioxidant activity and energy metabolism acceleration is unclear, so further studies about its mechanism are necessary.

In conclusion, we found that 3,4,5-tri-CQA has a neuroprotective effect on $A\beta_{1-42}$ treated SH-SY5Y cells. The mRNA expression of glycolytic enzymes (G3PDH and PGAM1) and the intracellular ATP level was increased in 3,4,5-tri-CQA treated cells. These findings suggest that 3,4,5-tri-CQA has a neuroprotective effect through the induction of G3PDH and PGAM1 expression and ATP production.

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