Protective Effect of Chlorogenic Acid against A β -Induced Neurotoxicity

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Abstract

Beta-amyloid (A β) is considered as one of the major causes of Alzheimer's disease. This study examined the neuroprotective effects of chlorogenic acid, a naturally occurring polyphenol which is distributed widely in plants, fruits and vegetables, against A β -induced toxicity. A β decreased significantly the viability of PC12 cells. This was accompanied by an increase in the intracellular calcium levels and cleaved caspase-3. In addition, A β induced an increase in Bax, and a decrease in Bcl-2 compared to the controls. However, a pre-treatment with chlorogenic acid rescued the PC12 cells from A β by attenuating the elevated intracellular calcium levels and reducing the levels of the apoptosis related proteins, including caspase-3, Bcl-2 and Bax. These results suggest that the protective effects of chlorogenic acid are, at least in parts, by attenuating the intracellular calcium influx and reducing apoptosis induced by A β .

Key Words: Chlorogenic acid, Beta-amyloid, Calcium influx, Apoptosis, PC12 cells

INTRODUCTION

The beta-amyloid peptide (A β) is a protein that is produced in excessive quantities and accumulates in the form of senile plaque in Alzheimer's disease (AD) (Kawahara and Kuroda, 2000). The sequential proteolysis of the amyloid precursor protein (APP) by β - and γ -secretases to A β and self-aggregation of A β monomers to oligomers are some of the characteristics found in AD (Selkoe, 1998; Lue *et al.*, 1999). A β -induced neurotoxicity is considered one of the major causes of AD (Cummins *et al.*, 1998; Kelly and Ferreira, 2006). In particular, the increased intracellular calcium levels are one of the mediators of A β -induced toxicity in neuronal cells (Fu *et al.*, 2006; Kelly and Ferreira, 2006). The apoptotic cell death induced by A β is also associated with the neuritic degeneration and the onset of AD (Loo *et al.*, 1993; Selkoe, 2000). In addition, the activation of caspase-3 plays a role in A β -induced apoptosis (Allen *et al.*, 2001) and an increase in activated caspase-3 has been reported in AD brains (Su *et al.*, 2001).

Chlorogenic acid (Fig. 1) is a naturally occurring polyphenol

that is distributed widely in plants, fruits and vegetables, such as coffee beans, potatoes and apples (Clifford, 1999; Zang *et al.*, 2003). It possesses a wide range of biological activities including anti-carcinogenic, anti-bacterial, anti-inflammatory and antioxidant activities *in vitro* (Huang *et al.*, 1988; Almeida *et al.*, 2006; dos Santos *et al.*, 2006). However, the possible beneficial effects of chlorogenic acid against A β have not been elucidated. Therefore, this study evaluated the neuroprotective effects of chlorogenic acid against A β -induced toxicity in PC12 cells (rat pheochromocytoma). To accomplish this, an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was carried out to determine if chlorogenic acid protected the PC12 cells against A β . To examine their underlying mechanisms, the effects of chlorogenic acid on the intracellular calcium level and apoptosis related proteins including Bcl-2, Bax and caspase-3 were evaluated as possible neuroprotective mechanisms against A β .

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Fig. 1. The structure of chlorogenic acid.

MATERIALS AND METHODS

Chemicals and reagents

MTT, sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO), absolute ethanol, 3-*tert*-butyl-4-hydroxyanisole (BHA), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma (St Louis, MO, USA). Chlorogenic acid was also obtained from Sigma. A β (25-35) was acquired from Bachem California Inc. (Torrance, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and horse serum (HS) were supplied by Invitrogen (Carlsbad, CA, USA).

Cell culture

PC12 cells were purchased from ATCC (Manassas, VA, USA) and maintained routinely in DMEM supplemented with 15% HS and 5% FBS, at 37°C under 5% CO₂. The cells were used for the experiments during the exponential growth phase.

Preparation of A β stock solution

The A β (25-35) used in this study was pre-aggregated prior to use because the A β oligomers are more toxic to neurons than monomers (soluble form) or fibrils (Kelly and Ferreira, 2006). Briefly, 1 mg A β was dissolved in 1 ml of DMEM and incubated in a 37°C water bath for three days to induce aggregation. The aggregated A β was then diluted to 100 μ g/ml (100 μ M) and stored at -20°C until needed because repeated freeze-thaw cycle of aggregated A β can reduce its neurotoxicity.

Determination of the ability of chlorogenic acid to protect PC12 cells against A β

The protection of PC12 cells from A β by chlorogenic acid was determined by measuring the potential of the cell to reduce MTT to MTT formazan, which indicates the cell viability (Park and Kim, 2002). Briefly, exponentially growing PC12 cells (4 $\times$ 10⁴ cells/well) were plated in 96-well tissue culture plates, after which the cells were pretreated with different concentrations of chlorogenic acid (1, 4, 20 and 100 μ g/ml) for 1 h. As a vehicle control, cells were treated with 0.1% DMSO. The cells were then incubated with A β (10 μ M) for an additional 24 h, after which a MTT solution (10 μ l/well, 5 mg/ml stock solution in phosphate buffered saline (PBS)) was added for 3 h at 37°C. The cells were then lysed in 100 μ l of a lysis buffer (10% w/v of SDS in 0.01N HCl) overnight at 37°C. The optical

density of the resulting solutions was determined colorimetrically at 570 nm using a microplate reader (Molecular Devices; Sunnyvale, CA, USA). The ability of chlorogenic acid (20 and 50 μ M) to protect the PC12 cells from different concentrations of A β (1, 5, 10, 25 and 50 μ M) was also determined using a MTT assay, as described above. Rosmarinic acid was used as the positive control (Iuvone *et al.*, 2006; Park *et al.*, 2009).

Determination of antioxidant activity of chlorogenic acid using DPPH

The antioxidant activity of chlorogenic acid was determined by evaluating its ability to scavenge the stable free radical, DPPH, into 1,1-diphenyl-2-picrylhydrazine (Smith *et al.*, 1987). Briefly, 99 μ l of DPPH (0.316 mM in ethanol) and 1 μ l of various concentrations of chlorogenic acid (1, 4, 20 and 100 μ g/ml) were mixed and incubated at 37°C for 30 min. The optical density was then measured at 517 nm using a microplate reader (Molecular Devices). Rosmarinic acid and were tested as the positive controls.

Effect of chlorogenic acid on the intracellular calcium level

The ability of chlorogenic acid to attenuate the A β -induced intracellular calcium levels was determined using Fura-2AM (Fura-2-acetoxymethyl ester, Invitrogen). Briefly, the PC12 cells were plated onto collagen-coated glass bottom dishes and loaded with Fura-2AM (final concentration 5 μ M) in a 10 mM HEPES buffer, containing 132 mM NaCl, 3 mM KCl, 10 mM glucose and 2 mM CaCl₂ (pH 7.4) for 1 h at room temperature. After the cells were rinsed with HEPES buffer, they were incubated at 37°C for 1 h. The change in the intracellular calcium levels was examined by fluorescence microscopy before and after the addition of A β and chlorogenic acid under the following conditions: excitation at 340 and 380 nm, and emission at 500 nm. The change in the intracellular calcium levels is expressed as the change in the fluorescence ratio at 340/380 nm.

Protein determination, electrophoresis and immunoblotting

Western blot analysis was performed to determine the effect of chlorogenic acid on the expression of the proteins related to apoptosis. Briefly, PC12 cells plated on 6-well tissue culture plates were pretreated with 20 or 50 μ M chlorogenic acid for 1 hr and followed by A β for another 24 hrs. To prepare the whole cell lysates, the cultures were washed twice with PBS, scraped in a Laemmli sample buffer and boiled immediately for 5 min. The protein concentration was then determined using the Lowry method. SDS-polyacrylamide gels were then run according to Laemmli (Laemmli, 1970), after which the protein was transferred to an Immobilon membrane (Millipore, Billerica, MA, USA). Immunodetection was performed using the method described in the literature (Park *et al.*, 2008) using the following antibodies: anti- α -tubulin (1:100,000, Sigma), anti-total caspase-3 (1:5,000, Cell Signaling, Denver, MA, USA), anti-cleaved caspase-3 (1:1,000, Cell Signaling), anti-Bax (1:1,000, Cell Signaling), and anti-Bcl-2 (1:1,000, Cell Signaling). Secondary antibodies conjugated to horseradish peroxidase (1:2,500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ, USA) were used to detect the proteins. Densitometry was then performed using

Fig. 2. The protective effect of chlorogenic acid (CHL) against $A\beta$ -induced cytotoxicity. (A) PC12 cells were incubated with $A\beta$ (10 μ M) in the presence or absence of chlorogenic acid (CHL, 2, 10, 50 or 250 μ M) for 24 h and the cell viability was estimated by a MTT assay. All data is reported as the mean $\pm$ SD of three independent experiments. * p <0.05, different from the $A\beta$ -treated groups. (B) PC12 cells were incubated with different concentrations of $A\beta$ in the presence or absence of chlorogenic acid (CHL, 2, 10, 50 or 250 μ M) for 24 h and the cell viability was then estimated by the MTT assay. Note that pretreatment with chlorogenic acid (CHL) significantly increased cell viability compared to $A\beta$ treatment (all doses). All data is reported as the mean $\pm$ SD of three independent experiments.

a Bio-Rad (Hercules, CA, USA) 700 flatbed scanner and Molecular Analyst software (Bio-Rad). The densitometric values were normalized using α -tubulin as the internal control. Scanning of the Western blots revealed the curve to be linear in the range used for each antibody.

Data analysis

All the data in the text and figures is expressed as the mean $\pm$ SD of at least three independent experiments. A one-way analysis of variance (ANOVA) followed by a Newman-Keuls post hoc test was performed to compare the groups. p -values <0.05 were considered significant.

RESULTS

Protection of PC12 cells against $A\beta$ -induced toxicity by chlorogenic acid

The neuroprotective effects of chlorogenic acid were evaluated by measuring the viability of the PC12 cells in the presence of $A\beta$ (10 μ M) with or without different concentrations of chlorogenic acid (2, 10, 50 or 250 μ M) using a MTT assay. Initially, exposure of PC12 cells to chlorogenic acid alone (1–100 μ g/ml) did not alter the cell viability compared to the DMSO-treated controls (data not shown). The neuroprotective ability of chlorogenic acid against $A\beta$ was evaluated using a MTT assay (Fig. 2A). As a result, $A\beta$ (10 μ M) induced approximately 50% cell death in the treated populations. However, pre-treatment of PC12 cells with 50 or 250 μ M chlorogenic acid attenuated the $A\beta$ -induced cytotoxicity significantly, whereas pre-treatment with low doses of chlorogenic acid (2 or 10 μ M) did not (Fig. 2A). The ED_{50} value of chlorogenic acid was 116.2 ± 9.5 μ M. As a positive control, the neuroprotective effects of rosmarinic acid against $A\beta$ were also tested. Rosmarinic acid at 18 ± 1.6 μ M protected 50% of the PC12 cells from $A\beta$ -induced toxicity. Compared to rosmarinic acid, chlorogenic acid showed relatively moderate neuroprotective effects against $A\beta$.

The neuroprotective effects of chlorogenic acid against dif-

Fig. 3. The antioxidant activity of chlorogenic acid (CHL). The antioxidant activity of chlorogenic acid (CHL, 2, 10, 50 and 250 μ M) was determined by evaluating its ability to scavenge the stable free DPPH radicals.

ferent concentrations of $A\beta$ (1, 5, 10, 25 or 50 μ M) were also determined using a MTT assay (Fig. 2B). Higher concentrations of $A\beta$ were more cytotoxic to the cells. A pre-treatment with chlorogenic acid (50 or 125 μ M) attenuated the $A\beta$ -induced cytotoxicity and increased the viability of PC12 cells at all $A\beta$ concentrations.

Antioxidant effect of chlorogenic acid

The antioxidant effects of chlorogenic acid were also examined to provide the relationship with the neuroprotective effects using DPPH. Rosmarinic acid and BHA were also tested as positive controls. As shown in Fig. 3, treatment of rosmarinic acid and BHA resulted in the scavenging of free radicals in a dose-dependent manners. Chlorogenic acid exhibited relatively low antioxidant activity compared to the positive controls, rosmarinic acid and BHA at 2, 10, and 50 μ M. However, the free radical scavenging effect of chlorogenic acid at 250 μ M was comparable to those of rosmarinic acid and BHA.

Fig. 4. Chlorogenic acid (CHL) prevented the increase in intracellular calcium levels induced by A β . A β -treated PC12 cells with or without chlorogenic acid (CHL, 50 or 125 μ M) were evaluated for changes in the intracellular calcium levels using Fura-2 AM. Pre-treatment with 50 or 125 μ M of chlorogenic acid attenuated the increase in intracellular calcium levels that was induced by A β (10 μ M). All data is reported as the mean $\pm$ SD of three different cultures, and at least 50 different cells from each culture. * p <0.05, different from the control group, # p <0.05, different from the A β -treated group.

Effect of chlorogenic acid on intracellular calcium levels

The effects of chlorogenic acid on the intracellular calcium levels induced by A β were evaluated using 5 μ M Fura-2AM. The cells treated with chlorogenic acid alone showed similar intracellular levels of calcium compared to DMSO-treated vehicle controls (data not shown). The cells treated with A β showed significantly higher intracellular calcium levels than the controls. However, a 1 h pre-treatment with 50 or 125 μ M of chlorogenic acid attenuated the increase in the intracellular calcium induced by A β (Fig. 4). Higher levels of chlorogenic acid (125 μ M) were more efficient in decreasing the intracellular calcium levels than lower levels of chlorogenic acid (50 μ M). However, no significant changes in the intracellular calcium levels were observed in the untreated or chlorogenic acid only-treated PC12 cells throughout the experimental period (data not shown). Overall, chlorogenic acid protects PC12 cells, in part, by attenuating the changes in the intracellular calcium levels induced by A β .

Anti-apoptotic effect of chlorogenic acid against A β

The anti-apoptotic effects of chlorogenic acid against A β were determined by measuring the changes in the anti-apoptotic and pro-apoptotic proteins by Western blotting analysis (Fig. 5A, B). The expression of the pro-apoptotic protein Bax was significantly higher in the cells treated with A β , whereas that of the anti-apoptotic protein, Bcl-2, was significantly attenuated. On the other hand, pre-treatment of the cells with chlorogenic acid significantly attenuated the increase in Bax and decrease in Bcl-2 induced by A β , and the Bcl-2/Bax ratio was increased significantly by a treatment with chlorogenic acid prior to A β . In addition, the effects of chlorogenic acid against A β in the expression of caspase-3 were also determined by Western blot analysis (Fig. 5A, C). Exposure of the cells to A β significantly increased the expression levels of cleaved caspase-3, whereas pre-treatment with chlorogenic acid attenuated the increase in cleaved caspase-3 induced by A β .

Fig. 5. Chlorogenic acid (CHL) reversed the levels of apoptotic proteins and caspase-3 induced by A β . (A) Western blot analysis of Bcl-2, Bax, cleaved caspase-3 and caspase-3 contents in the whole cell lysates of PC12 cells treated with A β (10 μ M) in the presence or absence of chlorogenic acid (CHL, 125 μ M) for 24 h using specific antibodies. (B, C) Graphs showing changes in the levels of Bcl-2/Bax ratio and cleaved caspase-3 in PC12 cells treated with A β in the presence or absence of chlorogenic acid (CHL, 125 μ M). An equal amount of the total proteins was loaded into each lane. The values are expressed as a percentage of the DMSO-treated controls. All data is reported as the mean $\pm$ SD of three different experiments. * p <0.05, different from the control groups, # p <0.05, different from the A β -treated groups.

DISCUSSION

The most well-known physiological role of chlorogenic acid, an ester of polyphenolic caffeic acid and cyclitol (-)-quinic acid, is antioxidant effect in vitro (Couladis *et al.*, 2002). Its antioxidant effect has been known to play a role in the prevention of Type 2 Diabetes Mellitus and cardiovascular disease (Paynter *et al.*, 2006).

One of the $\text{A}\beta$ -induced toxicity mediators is the increased intracellular calcium levels in neuronal cells (Fu *et al.*, 2006; Kelly and Ferreira, 2006). Therefore, the blockage of calcium influx induced by $\text{A}\beta$ might be a good therapeutic approach for providing neuroprotection against $\text{A}\beta$ -induced cell death. In mast cells, chlorogenic acid suppressed IgE-antigen complex-induced calcium uptake, which might be due to the decrease in mast cell activation (Qin *et al.*, 2010). Recently, the dysregulation of calcium recovery (calcium buffering capacity) induced by $\text{A}\beta$ was improved slightly by a treatment with chlorogenic acid (Joseph *et al.*, 2010). In the present study, chlorogenic acid at 50 $\mu\text{g}/\text{ml}$ significantly attenuated the increase in intracellular calcium levels induced by $\text{A}\beta$ in PC12 cells, suggesting that a decrease in calcium influx in PC12 cells is one of the possible mechanisms by which chlorogenic acid exerts its neuroprotective effect against $\text{A}\beta$.

The excess of $\text{A}\beta$ production and aggregation in the brain, and $\text{A}\beta$ -induced apoptotic cell death is considered to be associated with the neuritic degeneration and the onset of AD (Loo *et al.*, 1993; Selkoe, 2000). Furthermore, the $\text{A}\beta$ -induced apoptotic cell death in PC12 cells is associated with the decreased expression of the anti-apoptotic molecule Bcl-2 and the increased expression of the pro-apoptotic molecule Bax (Xiao *et al.*, 2002; Yuyama *et al.*, 2003). The activation of caspase-3 plays a role in $\text{A}\beta$ -induced apoptosis (Allen *et al.*, 2001) and an increase in activated caspase-3 has been reported in AD brains (Su *et al.*, 2001). Therefore, the decrease in Bax or active caspase-3 expression and increase in Bcl-2 expression can provide a neuroprotective effect by inhibiting $\text{A}\beta$ -induced apoptosis. For example, organic extracts of mulberry leaves containing mainly phenolic compounds, including chlorogenic acid, inhibited apoptosis in HepG2 hepatoma cells by arresting the cell cycle in the G2/M phase (Granado-Serrano *et al.*, 2007; Naowaratwattana *et al.*, 2010). In addition, chlorogenic acid exhibited an anti-apoptotic effect on methylglyoxal-induced neuroblastoma cell apoptosis (Neuro-2A cells) (Huang *et al.*, 2008). In this study, chlorogenic acid exhibited an anti-apoptotic effect by downregulating the expression of cleaved caspase-3 and Bax, and the upregulating Bcl-2. Therefore, the effects of chlorogenic acid can help inhibit $\text{A}\beta$ -induced apoptosis.

In summary, this study investigated the neuroprotective effects of chlorogenic acid against $\text{A}\beta$ in PC12 cells. Chlorogenic acid prevented the increase in intracellular calcium levels induced by $\text{A}\beta$. Furthermore, chlorogenic acid reduced the activation of caspase-3 and reversed the levels of Bcl-2 and Bax expression induced by $\text{A}\beta$. Overall, these results suggest that chlorogenic acid attenuates $\text{A}\beta$ -induced neurotoxicity by reducing apoptotic effect and inhibiting calcium influx by $\text{A}\beta$. Further in vivo studies are necessary to prove potential of chlorogenic acid as an anti-AD agent.

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