INTRODUCTION

The beta-amyloid peptide (Aβ) 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 Aβ and self-aggregation of Aβ monomers to oligomers are some of the characteristics found in AD (Selkoe, 1998; Lue et al., 1999). Aβ-induced neurotoxicity is considered one of the major causes of AD (Cummings et al., 1998; Kelly and Ferreira, 2006). In particular, the increased intracellular calcium levels are one of the mediators of Aβ-induced toxicity in neuronal cells (Fu et al., 2006; Kelly and Ferreira, 2006). The apoptotic cell death induced by Aβ 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 Aβ-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 Aβ have not been elucidated. Therefore, this study evaluated the neuroprotective effects of chlorogenic acid against Aβ-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 Aβ. To examine their underlying mechanisms, the effects of chlorogenic acid on the intracellular calcium level and apoptosis related proteins including caspase-3, Bcl-2 and Bax were evaluated as possible neuroprotective mechanisms against Aβ.

Abstract

Beta-amyloid (Aβ) 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 Aβ-induced toxicity. Aβ decreased significantly the viability of PC12 cells. This was accompanied by an increase in the intracellular calcium levels and cleaved caspase-3. In addition, Aβ 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 Aβ 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 Aβ.

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

PROTECTIVE EFFECT OF CHLOROGENIC ACID AGAINST Aβ-INDUCED NEUROTOXICITY

Chan Woo Lee¹, Tae Joon Won¹, Hak Rim Kim², Dongho Lee³, Kwang Woo Hwang¹,* and So-Young Park⁴,*

¹Immune Modulation Lab., College of Pharmacy, Research Institute for Translational System Biomics, Chung-Ang University, Seoul 156-756;
²Department of Pharmacology, College of Medicine, Dankook University, Cheonan 330-714;
³Division of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 136-713;
⁴Department of Pharmacognosy, College of Pharmacy, Dankook University, Cheonan 330-714, Republic of Korea

Abstract

Beta-amyloid (Aβ) 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 Aβ-induced toxicity. Aβ decreased significantly the viability of PC12 cells. This was accompanied by an increase in the intracellular calcium levels and cleaved caspase-3. In addition, Aβ 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 Aβ 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 Aβ.

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cells (4 × 10^4) produce MTT to MTT formazan, which indicates the cell viability was determined by measuring the potential of the cell to reduce MTT at 37°C. The cells were then lysed in 100 μl 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 μM) to protect the PC12 cells from different concentrations of Aβ (1, 5, 10, 25 and 50 μM) 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 μl of DPPH (0.316 mM in ethanol) and 1 μl of various concentrations of chlorogenic acid (1, 4, 20 and 100 μg/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 Aβ-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 μM) 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 Aβ 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 μM chlorogenic acid for 1 hr and followed by Aβ 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

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**Fig. 1.** The structure of chlorogenic acid.
a Bio-Rad (Hercules, CA, USA) 700 flatted 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± 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 Aβ-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 Aβ (10 μM) with or without different concentrations of chlorogenic acid (2, 10, 50 or 250 μM) using a MTT assay. Initially, exposure of PC12 cells to chlorogenic acid alone (1-100 μg/ml) did not alter the cell viability compared to the DMSO-treated controls (data not shown). The neuroprotective ability of chlorogenic acid against Aβ was evaluated using a MTT assay (Fig. 2A). As a result, Aβ (10 μM) induced approximately 50% cell death in the treated populations. However, pre-treatment of PC12 cells with 50 or 250 μM chlorogenic acid attenuated the Aβ-induced cytotoxicity and increased the viability of PC12 cells at all Aβ 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 the stable free DPPH radicals. 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 the stable free DPPH radicals. Chlorogenic acid exhibited relatively low antioxidant activity compared to the positive controls, rosmarinic acid and BHA at 2, 10, and 50 μM. However, the free radical scavenging effect of chlorogenic acid at 250 μM was comparable to those of rosmarinic acid and BHA.
Effect of chlorogenic acid on intracellular calcium levels

The effects of chlorogenic acid on the intracellular calcium levels induced by Aβ were evaluated using 5 μM 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 Aβ showed significantly higher intracellular calcium levels than the controls. However, a 1 h pre-treatment with 50 or 125 μM of chlorogenic acid attenuated the increase in intracellular calcium induced by Aβ (Fig. 4). Higher levels of chlorogenic acid (125 μM) were more efficient in decreasing the intracellular calcium levels than lower levels of chlorogenic acid (50 μM). 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 Aβ.

Anti-apoptotic effect of chlorogenic acid against Aβ

The anti-apoptotic effects of chlorogenic acid against Aβ 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 Aβ, 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 Aβ, and the Bcl-2/Bax ratio was increased significantly by a treatment with chlorogenic acid prior to Aβ. In addition, the effects of chlorogenic acid against Aβ in the expression of caspase-3 were also determined by Western blot analysis (Fig. 5A, C). Exposure of the cells to Aβ significantly increased the expression levels of cleaved caspase-3, whereas pre-treatment with chlorogenic acid attenuated the increase in cleaved caspase-3 induced by Aβ.
DISCUSSION

The most well-known physiological role of chlorogenic acid, an ester of polyphenolic caffeic acid and cymcit (l)-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 Aβ-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 Aβ might be a good therapeutic approach for providing neuroprotection against Aβ-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 Aβ was improved slightly by a treatment with chlorogenic acid (Joseph et al., 2010). In the present study, chlorogenic acid at 50 μg/ml significantly attenuated the increase in intracellular calcium levels induced by Aβ 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 Aβ.

The excess of Aβ production and aggregation in the brain, and Aβ-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 Aβ-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 Aβ-induced apoptosis (Allen et al., 2001) and an increased 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 Aβ-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 methylglyoxyl-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 Aβ-induced apoptosis.

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

REFERENCES


