Protective Effect of Chlorogenic Acid against Aβ-Induced Neurotoxicity

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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

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 Bcl-2, Bax and caspase-3 were evaluated as possible neuroprotective mechanisms against Aβ.

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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. Aβ (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 Aβ stock solution**

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

**Determination of the ability of chlorogenic acid to protect PC12 cells against Aβ**

The protection of PC12 cells from Aβ by chlorogenic acid was determined by measuring the potential of the cell to reduce MTT to formazan, which indicates the cell viability (Park and Kim, 2002). Briefly, exponentially growing PC12 cells (4×10⁵ 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 μg/ml) for 1 h. As a vehicle control, cells were treated with 0.1% DMSO. The cells were then incubated with Aβ (10 μM) for an additional 24 h, after which a MTT solution (10 μl/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 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 h 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...