Neuroprotective Effect of Honokiol and Magnolol, Compounds from *Magnolia officinalis*, on Beta-amyloid-induced Toxicity in PC12 Cells

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Amyloid β peptide (Aβ) induced toxicity is a well-established pathway of neuronal cell death which might play a role in Alzheimer’s disease. In this regard, the toxic effect of Aβ on a cultured Aβ-sensitive neuronal cell line was used as a primary screening tool for potential anti-Alzheimer’s therapeutic agents. The effects of nine pure compounds (vitamin E, α-asarone, salidroside, baicolin, magnolol, gastrodin, bilobalide, honokiol and β-asarone) from selected Chinese herbs on neuronal cell death induced by Aβ in NGF-differentiated PC12 cells were examined. Only two of the studied compounds, honokiol and magnolol, significantly decreased Aβ-induced cell death. Further experiments indicated that their neuroprotective effects are possibly mediated through reduced ROS production as well as suppression of intracellular calcium elevation and inhibition of caspase-3 activity. The results provide for the first time a scientific rationale for the clinical use of honokiol and magnolol in the treatment of Alzheimer’s disease. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: Aβ; neurotoxicity; Alzheimer’s disease; neuroprotective; calcium; apoptosis.

INTRODUCTION

Alzheimer’s disease (AD) is the leading cause of dementia. Pathological hallmarks of AD include neuronal degeneration and senile plaques (Vickers *et al.*, 2000). Such plaques are composed of compacted amyloid β peptide (Aβ), which is a 40–43 amino acid peptide derived from the endoproteolytic cleavage of amyloid precursor protein (APP) (Selkoe, 1998). Aβ aggregates into amyloid fibrils, which have been reported to be neurotoxic in vitro (Munoz and Inestrosa, 1999) and in vivo (Soto *et al.*, 1998). Thus, modulation of Aβ toxicity has been speculated to be a potential therapy for AD. In this regard, the toxic effect of Aβ on a cultured neuronal cell line can be used as a primary screening assay in the search for potential leads for the treatment of AD.

For millennia, Chinese physicians have used many herbs to treat dementia, and these effects were documented in Chinese herbal books (Oishi *et al.*, 1998). Herbal drugs showing antidepressive, antiinflammatory, antioxidant and/or antipsychotic benefits may be particularly beneficial to AD patients because, in the late stages of disease progression, AD patients exhibit psychotic changes in addition to cellular changes relating to inflammation, oxidation and infection.

The aim of the present study was to explore the potential of compounds from selected Chinese herbs for their capacities to protect rat adrenal medulla pheochromocytoma PC12 cells from the cytotoxic effect of Aβ. Furthermore, the study explored whether these compounds could reverse changes in calcium levels, reactive oxygen species (ROS) and neuronal apoptosis associated with Aβ neurotoxicity.

MATERIAL AND METHODS

Material. The chemicals used in this study were as follows: All the materials for cell culture were purchased from Gibco BRL (Carlsbad, CA, USA) and Life Technologies (Grand Island, NY, USA). Honokiol and magnolol were purchased from Wako Pure Chemical Industries (Los Angeles, CA, USA), and other pure compounds (including bilobalide, vitamin E (α-tocopherol), α-asarone, β-asarone and baicolin), unless otherwise stated, were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Gastrodin and salidroside were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, China). Aβ1–40 and EnzoLyte™ AMC caspase-3 assay kit were purchased from Anaspec (San Jose, CA, USA). [3-(4,5)-dimethylthiazol-2-yl]-2,5-di-phenyltetrazolium (MTT) and 2’,7’-dichlorofluoresceindiacetate (H2DCF-DA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). NFG was purchased from Gibco BRL (Carlsbad, CA, USA). Fluo-3 AM was purchased from Molecular Probes, Inc. (Eugene, OR, USA). All other chemicals and solvents were of analytical or high performance liquid chromatography (HPLC) grade, and all water used was deionized and double-distilled.

Cell culture. Rat pheochromocytoma cells (PC12 cells) were obtained from the American Type Culture Collection (ATCC, CRL 1721) and were grown in Dulbecco’s
modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum, 10% horse serum, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in 5% CO₂/95% air. PC12 cells were split at a density of 1 x 10⁴ cells/100 µL, plated into 96-well plates at 100 µL/well, and allowed to adhere for 24 h at 37°C. PC12 cells were differentiated with 50 ng/mL NGF in DMEM. Five days after NGF addition, the medium was replaced with DMEM containing 50 ng/mL NGF with or without AB (dissolved in pyrogen-free water) plus different concentrations of pure compounds. The pure compounds were diluted in DMSO to different concentrations. The final DMSO concentration in each sample was 0.1%, and this concentration did not affect cell growth or death.

**MTT cell viability assay.** A methylthiazol tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT) conversion assay was used to determine cell viability under each treatment condition (Mosmann, 1983). The MTT assay relies primarily on the mitochondrial metabolic capacity of viable cells and reflects the intracellular redox state. After incubation, the cells were treated with the MTT solution (final concentration: 1 mg/mL) for 3 h. The blue formazan crystals formed in intact cells were solubilized with lysis buffer (20% (v/v) sodium dodecylsulfate in 50% (v/v) aqueous N,N-dimethylformamide with an adjusted pH of 4.5). The optical density of each well was measured with a 96-well microplate spectrophotometer (Bio-Rad, Hercules, CA, USA, Benchmark) at the test wavelength of 570 nm.

**Measurement of intracellular calcium levels.** Changes in intracellular free calcium levels ([Ca²⁺]) in a suspension of PC12 cells were determined by the Fluo-3 method (Boldyrev et al., 2000; Wang and Xu, 2005). After exposure to ABβ₄₀ at various concentrations for various times with different concentrations of pure compounds, NGF-differentiated PC12 cells were then loaded with the Ca²⁺-sensitive fluorescent dye Fluo-3 AM (5 µM) at 37°C for 30 min in culture medium and then washed free of extracellular Fluo-3 AM dye and resuspended in Krebs-Ringer-HEPES (KRH) buffer (131 mM NaCl, 5 mM KCl, 1.3 mM MgSO₄, 1.3 mM CaCl₂, 0.4 mM KH₂PO₄, 6 mM glucose, 20 mM HEPES, pH 7.4). Fluorescence was determined using a microplate spectrophotometer (Automed Multifunctional Monochromator Reader, Tecan-B017554, Safire Tecan-F129013, Tecchomp Ltd) with excitation and emission wavelengths of 488 nm and 525 nm, respectively. The results are the mean ± SEM of quadruplicate measurements from three separate experiments and are expressed as the percentage increase in relative fluorescence units (RFU) relative to the reading at baseline.

**Data analysis.** Statistical analysis of the data for multiple comparisons was performed by analysis of variance (ANOVA). For single comparisons, the significance of differences between means was determined by Student’s t-test. A value of p < 0.05 was regarded as statistically significant.

**RESULTS**

**Effect of AB peptide on cytotoxicity in NGF-differentiated PC12 cells**

The toxicity of ABβ on NGF-differentiated PC12 cells was assessed by MTT assay. It was confirmed that when NGF-differentiated PC12 cells were exposed to ABβ₄₀ (0.1 µM, 0.5 µM, 1 µM, 2.5 µM, 5 µM or 10 µM) for 24 h, the rate of MTT reduction was decreased in a concentration-dependent manner (Fig. 1), as reported in a previous study (Ito et al., 2003). Therefore, a fixed concentration of ABβ₄₀, 2.5 µM, was used for the determination of ABβ₄₀-induced PC12 cell damage in the present experiments.

MTT cell viability assay

The MTT assay was also used to measure protective effects of compounds on cell viability. PC12 cells were incubated in culture medium containing 2.5 μM Aβ1–40 with or without the following compounds: vitamin E, α-tocopherol, α-asarone, salidroside, baikolin, magnolol, gastrodin, bilobalide, honokiol and β-asarone. As shown in Fig. 2, among the selected molecules tested, only honokiol, magnolol and vitamin E significantly protected PC12 cells from the cytotoxic effect of Aβ1–40. After 24 h exposure to 2.5 μM Aβ1–40 alone, the degree of reduction of MTT by PC12 cells was decreased by 63.1 ± 2.6%, and honokiol, magnolol and vitamin E significantly increased cell viability. Further analysis revealed that the protective effects of honokiol and magnolol were significantly better than vitamin E (Fig. 2).

Measurement of reactive oxygen species (ROS)

By using the ROS fluorescent dye, DCF, it was found that exposure of NGF-differentiated PC12 cells to 2.5 μM Aβ1–40 for 24 h resulted in a highly significant increase (256%) in DCF fluorescence. Incubation of NGF-differentiated PC12 cells with vitamin E, honokiol or magnolol (100 μM, 50 μM, 10 μM, 1 μM) significantly inhibited Aβ-induced increase in ROS accumulation in a dose dependent manner (Fig. 3).

Intracellular calcium levels

Since the mechanism of toxicity of aged Aβ1–40 may be mediated, in part, by elevations of [Ca2+]i (Brorson et al., 1995; Mattson, 2002), the study examined whether honokiol and magnolol affected the Aβ-induced increase of [Ca2+]i (Fig. 4). Exposure of NGF-differentiated PC12 cells to Aβ1–40 for 24 h resulted in a 130% elevation of [Ca2+]i. The effects of honokiol and magnolol were assessed at this time point. Results showed that Aβ-induced changes in [Ca2+]i were reduced by honokiol, magnolol and vitamin E.

Measurement of caspase-3 activity

This study evaluated caspase activity by measuring relative levels of activated caspase-3 using the fluorogenic caspase-3 substrate DEVD-AMC. Caspase-3 was activated in PC12 cells by exposure to Aβ. As shown in Fig. 5, caspase-3 activity nearly doubled after 24 h incubation with 2.5 μM Aβ1–40. When honokiol or magnolol were included, the caspase-3 activity was reduced in a dose dependent manner (Fig. 5). These results suggest that apoptosis in Aβ-treated PC12 cells is mediated by caspase-3, and the protective action of honokiol and magnolol may, at least in part, be attributed to inhibition of the caspase cascade.

DISCUSSION

Aβ is associated with senile plaques in the brains of Alzheimer’s patients, and can be cytotoxic to cultured neurons (Cotman and Su, 1996; Bayer et al., 2001). Aβ1–40...
is the major species of Aβ secreted from cultured cells and in cerebrospinal fluid (CSF); in addition, Aβ1–40 forms calcium-permeable channels in the cell membrane and subsequently induces membrane injury (Mattson, 2002). Therefore, Aβ1–40 was used in our experiment. It confirmed the findings of a previous study that NGF-differentiated PC12 cells, a neuron-like cell line, exhibit MTT reduction which is decreased with exposure to increasing concentrations of Aβ1–40 (Ito et al., 2003).

To search for potential antidementia agents, vitamin E and eight pure compounds were selected and screened from selected herbs which have traditionally been used for the treatment of senile dementia. These pure compounds from selected herbs included honokiol and magnolol from Magnolia officinalis, α-asarone and β-asarone from rhizoma acori graminei, baicolin from scutellariae radix, gastrodin from rhizoma gasterodae, sairdoside from Rhodiola rosea, and biloba from Ginkgo biloba. Among them, it was demonstrated that honokiol and magnolol can protect PC12 cells from the cytotoxic effect of Aβ1–40. Honokiol and magnolol are two prominent constituents isolated from the herb Magnolia officinalis, which is used in the treatment of a number of neurological diseases in traditional Chinese medicine (Watanabe et al., 1983). Previous studies have reported the antioxidant effect of honokiol and magnolol (Lo et al., 1994). However, no previous studies reported whether honokiol and magnolol can protect neurons from the cytotoxicity of Aβ. The result, although preliminary, provides for the first time a scientific rationale for the clinical use of honokiol and magnolol in the treatment of AD. Although the mechanisms of the neurotoxicity exerted by aggregated Aβ are still not fully known, these may include the generation of ROS, an increase in intracellular Ca2+ concentration, and induction of apoptosis (Mattson, 2002). Honokiol and magnolol might exert neuroprotective effects by these mechanisms.

Several investigators have demonstrated that the neurotoxicity of Aβ can be mediated by ROS, which may contribute to the increased evidence of apoptosis found in AD (Behl et al., 1994; Markesbery, 1997). The ROS generated by Aβ toxicity causes damage in AD through different mechanisms, including membrane lipid peroxidation, receptor mediated mechanisms and disruption of cellular calcium homeostasis (Dyrks et al., 1992; Wang and Xu, 2005). The results have confirmed previous reports that Aβ-induced PC12 cells produce ROS and can be protected from Aβ toxicity by antioxidant and free radical scavengers such as vitamin E (Pereira et al., 1991; Behl et al., 1992). Moreover, it was shown for the first time that honokiol and magnolol reduced, in a concentration-dependent manner, the production of ROS induced by Aβ1–40. Since antioxidants are known to attenuate Aβ-induced oxidative injury, it is likely that the previously reported antioxidant properties of honokiol and magnolol could contribute to its beneficial effect (Cash et al., 2002).

Previous investigators have shown that Aβ-induced neurotoxic actions in PC12 cells are associated with an elevation of [Ca2+], and that this effect is blocked by antioxidants such as vitamin E (Mattson, 2002). In addition, it has been suggested that the increase in [Ca2+] induced by Aβ in PC12 cells is not the result of an influx of calcium through voltage-dependent calcium channels, but rather it is the result of a free radical-mediated process (Zhou et al., 1996). In the present study, it was shown that exposure of NGF-differentiated PC12 cells

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**Figure 3.** Effect of honokiol and magnolol on Aβ1–40 (2.5 μM)-induced formation of reactive oxygen species (ROS). ROS formation, evaluated by the oxidation of 2′,7′-dichlorofluorescein (H2DCF) to the fluorescent 2′,7′-dichlorofluorescein (DCF), was assessed 24 h after incubation with Aβ1–40. The results are the mean ± SEM of quadruplicate measurements from three separate experiments. ***p < 0.001, **p < 0.01, *p < 0.05 compared with cells treated with only Aβ1–40. Honokiol and magnolol are two prominent constituents isolated from the herb Magnolia officinalis, which is used in the treatment of a number of neurological diseases in traditional Chinese medicine (Watanabe et al., 1983). Previous studies have reported the antioxidant effect of honokiol and magnolol (Lo et al., 1994). However, no previous studies reported whether honokiol and magnolol can protect neurons from the cytotoxicity of Aβ. The result, although preliminary, provides for the first time a scientific rationale for the clinical use of honokiol and magnolol in the treatment of AD. Although the mechanisms of the neurotoxicity exerted by aggregated Aβ are still not fully known, these may include the generation of ROS, an increase in intracellular Ca2+ concentration, and induction of apoptosis (Mattson, 2002). Honokiol and magnolol might exert neuroprotective effects by these mechanisms.

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to Aβ1–40 for 24 h resulted in an approximately 3.5-fold elevation of [Ca2+]i. The results further revealed that honokiol and magnolol counteracted the Aβ1–40-induced elevation of [Ca2+]i, which is in agreement with the anti-oxidant properties of honokiol and magnolol reported here and elsewhere (Lo et al., 1994).

Multiple lines of evidence have implicated Aβ as neurotoxic, causing neuronal cells to enter apoptosis (Martin et al., 2001; Troy et al., 2001). Apoptosis is associated with the activation of a family of aspartic acid-specific cysteine proteases, referred to as caspases (Nicholson and Thornberry, 1997). In the caspase family, which consists of more than 10 homologues, caspase-3 has been suggested to play an important role in Aβ-induced apoptosis in vitro (Harada and Sugimoto, 1999). In addition, analysis of the apoptotic cascade in Aβ-induced cell death revealed significant cellular loss in the hippocampal regions of wild-type mice and dramatic rescue of neuronal cell death in caspase-3 deficient mice, indicating that Aβ-induced neuronal death is mediated in vivo as well as in vitro by the caspase-3 apoptotic cascade (Takuma et al., 2004). In the present study, it was shown that honokiol and magnolol can inhibit the elevation of caspase-3 activity in Aβ1–40-treated PC12 cells, suggesting that honokiol and magnolol could exert a protective role at the execution phase of apoptosis.

In summary, the present study demonstrated that compounds from Magnolia officinalis, honokiol and magnolol, significantly decreased Aβ-induced cell death. Their neuroprotective effects may be due to decreases in reactive oxygen species (ROS) production, intracellular calcium and caspase-3 activity. Thus, the use of honokiol and magnolol, or their analogs, should be explored as a therapeutic approach for AD.

Acknowledgements

Funding was provided by the School of Pharmacy, the Chinese University of Hong Kong.

Conflict of Interest

The authors have declared that there is no conflict of interest.

REFERENCES


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