Protective Effects of Decursin and Decursinol Angelate against Amyloid β-Protein-Induced Oxidative Stress in the PC12 Cell Line: The Role of Nrf2 and Antioxidant Enzymes

Li Li, Wei Li, Sang-Won JUNG, Yong-Woo LEE, and Yong-Ho KIM†

Department of Smart Foods and Drugs, Graduate School, Inje University, Gimhae 621-749, South Korea

Received August 20, 2010; Accepted December 8, 2010; Online Publication, March 7, 2011
[doi:10.1271/bbb.100606]

The protective effects of decursin (D) and decursinol angelate (DA) purified from Angelica gigas Nakai on amyloid β-protein (Aβ)-induced neurotoxicity and the underlying mechanisms were investigated. Aβ plays a major role in the pathogenesis of Alzheimer’s disease (AD) by eliciting oxidative stress. It significantly increased cytotoxicity and lipid peroxidation, but decreased glutathione contents and antioxidant enzyme activities. All of these results were markedly reversed by pretreatment with D or DA. Nuclear transcription factor Nrf2, which regulates the expression of antioxidant enzymes, was significantly increased by D or DA pretreatment. Furthermore, D and DA suppressed Aβ aggregation. These results suggest that D and DA increase cellular resistance to Aβ-induced oxidative injury in the rat pheochromocytoma (PC12) cells, presumably through not only the induction of Nrf2 and related antioxidant enzymes, but also the anti-aggregation of Aβ. Thus D and DA have therapeutic potential in treating AD and other oxidative stress-related diseases.

Key words: decursin; decursinol angelate; amyloid β-protein; NF-E2-related factor-2; antioxidant enzymes

Alzheimer’s disease (AD) is the most common form of senile dementia, affecting millions of people worldwide. It is characterized by progressive degeneration and loss of neurons in the brain, correlated with the appearance of neurofibrillary tangles and senile plaques, the two neuropathological hallmarks of AD.1) Amyloid β-peptide (Aβ) is the major component of senile plaques, and is considered to have a causal role in the development and progress of AD. There is evidence indicating that oxidative stress is a critical event in the pathogenesis of AD. This hypothesis is supported by studies using postmortem brain tissue from patients with AD2,3) and by in vitro studies.4) Oxidative stress can cause cell death by damaging cellular macromolecules, such as lipids, proteins, and nucleic acids. The brain is especially sensitive to oxidative stress, owing to high oxygen consumption and high levels of polyunsaturated fatty acids, making it particularly vulnerable to lipid peroxidation. In AD, oxidative stress is suspected to be generated by Aβ.5,6)

NF-E2-related factor-2 (Nrf2), a member of the basic leucine zipper (bZIP) transcription factor family, resides in the cytoplasm bound to its inhibitor protein, Keap 1. It translocates to the nucleus after stimulation. It is a potent activator of antioxidant response element (ARE)-mediated gene expression, moderating the transcriptional induction of a battery of genes encoding for antioxidative enzymes and cytoprotective proteins.5,6) Hence Nrf2 is presumably one of the most important pathways involved in cellular protection against xenobiotics and oxidative stress.7,8) Neural cells from nrf2-knockout mice were more vulnerable to oxidative stress than those from nrf2 wild-type mice.9,10) In addition, overexpression of Nrf2 protein dramatically increased the resistance of neurons to oxidative cell death.11) More importantly, upregulation of Nrf2-driven antioxidant enzymes is beneficial in in vitro and in vivo models of neurodegenerative diseases.12–16)

Angelica gigas Nakai (Umbelliferae) root is used in traditional oriental herbal medicine to treat female afflictions, and is regarded by herbalists as female ginseng for its hemopoietic and health-promoting activities.17) There are reports on the pharmacological properties of this plant, showing anti-cancer,18–22) anti-bacterial,23) anti-platelet aggregation,24) anti-nematodal,25) and antioxidant activities,26) generally attributed to the major active ingredients, decursin (D) and decursinol angelate (DA).27,28) Structural isomers on the side chain. Recently, it has been reported that D and DA exhibit potent neuroprotective activity against glutamate-induced neurotoxicity in primary cultures of rat cortical cells29) and greatly improve scopolamine-induced amnesia in mice,30,31) but few studies of the role of Nrf2 in the D and DA protective effect against Aβ-induced oxidative stress have been conducted. In order to evaluate the neuroprotective activity of D and DA, we sought to determine whether they would protect against Aβ25,35-induced oxidative stress using the rat PC12 pheochromocytoma cell line as an in vitro model.

Abbreviations: Aβ, amyloid β-protein; APP, amyloid precursor protein; ARE, antioxidant response element; AD, Alzheimer’s disease; CAT, catalase; D, decursin; DA, decursinol angelate; GAFDH, glyceraldehydes-3-phosphate dehydrogenase; GR, glutathione reductase; GSH, glutathione; Gpx, glutathione peroxidase; GSSG, oxidized glutathione; GST, glutathione-S-transferase; HS, horse serum; MDA, malondialdehyde; Nrf2, NF-E2-related factor-2; PC12, the rat pheochromocytoma; PD, Parkinson’s disease; SOD, superoxide dismutase; TBS, Tris-buffered saline; ThT, Thioflavin-T; TNB, 5-thio-2-nitrobenzoic acid
Materials and Methods

Materials. Amyloid beta-protein (25–35) trifluoroacetic acid salt (Ap25-35) was from Bachem California (Torrance, CA). RPMI-1640, GlutaMAX™-I, penicillin-streptomycin, fetal bovine serum (FBS), and horse serum (HS) were purchased from Invitrogen (Grand Island, NY). A BCA™ protein assay kit was from ThermoFisher Scientific (Barrington, IL). Assay kits for cytotoxicity (WST-8), superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione (GSH) were from Cayman Chemical Company (Ann Arbor, MI). A lipid peroxidation colorimetric assay kit was from Biochrome Biochemical Research (Rochester Hills, MI). Antibodies to Nrf2 (C-20) were from Santa Cruz Biotechnology (Santa Cruz, CA), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) rabbit mAb and anti-rabbit IgG alkaline phosphatase (AP)-linked antibodies were from Cell Signaling Technology (Danvers, MA). All the other reagents were of the highest grade and were from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Preparation of decursin and decursinol angelate. D and DA were purified from the ethanol extract by recycling the manufacturer’s instructions (Cayman Chemical Company). Cells were treated with various chemotherapeutics followed by exposure for 3 h to various concentrations of D and DA, diluted into serum-free medium to the desired concentrations immediately before use.

Preparation of Aβ25-35 stock solution. Aβ25-35, the most toxic peptide fragment derived from the amyloid precursor protein (APP), was dissolved in deionized distilled water at a concentration of 1 nmol, and was incubated at 37 °C for 3 d to induce maximal aggregation, according to a previous report. In order to create stable conditions for the aged stock solution, the solution was stored at –80 °C and diluted in serum-free medium to the desired concentrations immediately before use.

Cell culture. The rat pheochromocytoma (PC12) cell line was a kind gift from Dr. K.-Y. Kam (Inje University). All cells were plated in poly-l-lysine coated culture dishes in RPMI-1640+GlutaMAX™-I containing 5% FBS, 10% HS, and 1% penicillin-streptomycin. The cells were grown at 37 °C in a humidified 5% CO2 environment, and the medium was changed every other day. Exponentially growing cells were utilized in the experiments.

PC12 cells were plated at appropriate densities according to the experimental scales. After the cells were attached, they were switched to serum-free medium for treatment. They were incubated with and without Aβ25-35 for 24 h, followed by exposure for 3 h to various concentrations of D and DA, diluted in serum-free medium. Then they were washed twice with cold PBS and harvested into ice-cold PBS and homogenized. The homogenate was centrifuged at 4 °C at 10,000 g for 30 min, and then the supernatant was ready for assessment.

Assay of cell viability with WST-8 cell proliferation assay kit. Cell viability was assessed with a WST-8 cell proliferation assay kit following the manufacturer’s instructions (Cayman Chemical Company, Ann Arbor, MI). Briefly, PC12 cells were seeded in 96-well culture plates.

After incubation, the media was supplemented with 10 μL/well WST for 2 h prior to spectrophotometric evaluation. Conversion of WST to formazan was measured at 450 nm with a fluorescence multi-detection reader (Synergy HT, Biotek, Highland Park, IL). This reaction reflects the reductive capacity of the cells, representing their viability, and the results were expressed as percentage of the control (untreated) cells. Decreased WST reduction was taken as an indication of neuronal cell injury.

Assessment of lipid peroxidation. The content of malondialdehyde (MDA), a compound produced during lipid peroxidation, was determined with a commercially available colorimetric assay kit (Oxford Biochemical Research, Rochester Hills, MI). This assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1), with MDA at 45 °C. One molecule of MDA reacts with two molecules of Reagent R1 to yield a stable chromophore with maximal absorbance at 586 nm. The level of MDA is expressed as μM.

Assay of glutathione content. GSH content was assayed following the instructions for the reagent kit (Cayman Chemical Company, Ann Arbor, MI). To quantify the GSH, an enzymatic recycling method using glutathione reductase was utilized. The sulfhydryl group of GSH reacts with DTNB (5,5-dithio-bis-2-nitrobenzoic acid, Eilman’s reagent) and produces a yellow 5-thio-2-nitrobenzoic acid (TNB). Mixed disulfide, GSTNB (between GSH and TNB), which is concomitantly produced, is reduced by glutathione reductase to recycle the GSH, and it produces more TNB. The rate of TNB production is directly proportional to this recycling reaction, which in turn is directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 405 or at 412 nm provides an accurate estimation of GSH in the sample.

Measurement of activities of the antioxidant enzymes. Superoxide dismutase (SOD), Glutathione peroxidase (GPx), catalase (CAT), and glutathione-S-transferase (GST) activities were measured following the protocols of commercially available kits (Cayman Chemical Company). SOD activity was evaluated utilizing a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. SOD activity was standardized by cytochrome c and xanthine oxidase coupled assay. GPx activity was measured indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of an organic hydroperoxide by GPx, was recycled to its reduced state by GR and NADPH. The oxidation of NADPH to NADP+ was accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A340 was directly proportional to the GPx activity in the sample. One unit was defined as the amount of enzyme that causes the oxidation of 1.0 nmol of NADPH to NADP+ per min at 25 °C. CAT activity was based on the rate of the enzyme with methanol in the presence of an optimal concentration of H2O2. The formaldehyde produced was measured spectrophotometrically with 4-amin-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald forms specifically a bicyclic heterocycle with aldehydes, which upon oxidation changes from colorless to a purple color. The level of CAT is expressed in units of the amount of enzyme that causes the formation of 1.0 nmol of formaldehyde per min at 25 °C. GST activity was measured by the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH). Conjugation was accompanied by an increase in absorbance at 340 nm. The rate of increase was directly proportional to the GST activity in the sample. One unit of enzyme conjugates 1.0 nmol of CDNB with reduced glutathione per min at 25 °C.

Assay of Aβ25-35 aggregation. The degree of Aβ aggregation was monitored by fluorescence after the addition of Thioflavin-T (ThT). ThT is a fluorescent dye that interacts with fibrils and oligomers having a crossed β-sheet structure. This process is accompanied by a characteristic increase in fluorescence intensity at 480 nm. Aβ25-35 was incubated in deionized distilled water with and without D and DA (0.01–10 μM) at 37 °C. Aliquots of 10 μL were drawn from the incubated samples at intervals of 12 h for 3 d, and were diluted into 190 μL of ThT in glycyne-NaOH. Fluorescence was measured in 96-well black plates with a fluorescence multi-detection reader (Synergy HT, Biotek), with excitation at 480 nm and emission at 482 nm. ThT fluorescence was normalized against the Aβ9 sample.

Nuclear protein isolation. Cells were treated with various chemicals, as detailed in the various figure legends. Nuclear extracts were isolated as described previously, with modifications. Briefly, cells were washed with cold PBS and resuspended in cold buffer A (lysis buffer, 20 mM N-2-hydroxyethylpiperezine-N′-2-ethanesulfonic acid or HEPES, pH 8.0, 1 μM ethylenediaminetetraacetic acid, 1.5 μM MgCl2, 10 μM KCl, 1 μM DTT, 1 μM sodium orthovanadate, 1 μM NaF, 1 μM PMSF, 0.5 mg/mL benzamidine, 0.1 mg/mL leupeptin, and 1.2 mg/mL aprotinin). They were allowed to swell on ice for 15 min. Then 7.5 μL of 10% v/v NP-40 was added and mixed vigorously by vortex for 10 s. The homogenate was centrifuged for 50 s at 16,000 g.
The nuclear pellet was resuspended in cold buffer B (extraction buffer, the lysis buffer containing 20% glycerol). All the protein fractions were stored at −70 °C until use, and the protein concentrations were measured with a BCA™ protein assay kit (ThermoFisher Scientific, Barrington, IL).

Western blot analysis. Western blotting was performed by the standard method. Equal amounts of proteins were fractionated by 10% SDS-polyacrylamide gel electrophoresis and electro-transferred to an Immun-Blot™ PVDF membrane (0.2 µm pore size, Bio-Rad). Membranes were blocked overnight at 4 °C in Tris-buffered saline (TBS), 0.05% v/v Tween-20, 150 mM NaCl, and 5% v/v Bovine Serum Albumin (BSA, Santa Cruz Biotechnology, Santa Cruz, CA), followed by 2 h of incubation with primary antibody diluted in the same buffer (Neu2 1:250, GAPDH 1:1000). After washing with 0.1% v/v Tween-20 in TBS, the membrane was incubated with anti-rabbit IgG AP-linked secondary antibody for 1 h at room temperature and then washed with the same buffer. The immune-blotted membrane was developed with 5-bromo-4-chloro-3-indoyl phosphate (BCIP)/nitroblue tetrazolium (NBT) color-developing solution. The blots in the samples were quantified by densitometry analysis using PDQuest software (version 7.0, Bio-Rad, Hercules, CA). All data for three independent experiments were expressed as relative intensity compared to the control group for statistical analysis.

Statistical analysis. Data bars represent the means ± SD (standard deviations) for at least three independent experiments in all cases. Two group comparisons were evaluated by Student’s t-test as appropriate. Differences were considered statistically significant when the p value was <0.05.

Results

D and DA protected PC12 cells against Aβ-induced toxicity

The toxicity of D and DA and their neuroprotective effects against Aβ-induced cytotoxicity were determined by measuring the viability of PC12 cells using a WST-8 Cell Proliferation Assay Kit (Cayman Chemical Company, Ann Arbor, MI). As shown in Fig. 1A, treatment with D or DA alone (0.01–10 µM) did not show any cytotoxicity as compared to the untreated control cells. However, treatment with a high dose of D or of DA (50 µM) reduced the viability of the PC12 cells. Treatment with Aβ25–35 (25 µM) for 24 h induced approximately 60% cell death. Hence that concentration was used in the present study (Fig. 1B). PC12 cells were treated with various sub-lethal concentrations of D and DA for 3 h, followed by further incubation for 24 h in the presence and the absence of Aβ25–35 (25 µM). Inclusion of D and DA at sub-lethal concentrations in the cell culture medium for 3 h before and during exposure to Aβ25–35 (25 µM) resulted in marked enhancement of survival of the PC 12 cells as compared to the Aβ25–35-treated group. Maximum rescue occurred at a concentration of 10 µM of D or DA (Fig. 1B). These results indicate that Aβ25–35 treatment significantly reduced the viability of PC12 cells, and that D and DA blocked the injury caused by Aβ25–35.

D and DA reduced the lipid peroxidation induced by Aβ in PC12 cells

Malondialdehyde (MDA) is a decomposition product of peroxidised polyunsaturated fatty acids, which is assessed as an index of lipid peroxidation. As shown in Fig. 2, when PC12 cells were exposed to Aβ25–35 (25 µM) for 24 h, a significant increase in the level of MDA was found. The levels of MDA slightly decreased, but were not significantly changed by pretreatment with lower concentrations (0.01–0.1 µM) of D or DA, while pretreatment with higher concentrations (1.0–10 µM) caused a significant decrease in MDA production in PC12 cells.

Fig. 1. Protective Effects of D and DA on Aβ25–35-Induced Cytotoxicity in PC12 Cells.
A. PC12 cells were treated with various concentrations of D and DA for 24 h, after which cell viability was estimated by WST-8 assay. B. PC12 cells were pretreated with various concentrations of D and DA for 3 h and then incubated with and without 25 µM Aβ25–35 for 24 h, after which cell viability was determined by WST-8 assay. Data are expressed as percent of values in untreated control cultures, and represent the mean ± SD for three experiments in triplicate. *p < 0.05 compared with control. #p < 0.05 compared with the group treated with Aβ25–35 alone. ##p < 0.01 compared with the group treated with Aβ25–35 alone.

Fig. 2. Effects of D and DA on Aβ25–35-Induced Lipid Peroxidation in PC12 Cells.
PC12 cells were pretreated with various concentrations of D and DA for 3 h and then incubated with and without 25 µM Aβ25–35 for 24 h. Lipid peroxidation was determined by measuring the levels of malondialdehyde formed. Data represent mean ± SD for three experiments in triplicate. *p < 0.05 compared with control. #p < 0.05 compared with the group treated with Aβ25–35 alone.
No significant changes were observed in the formation of MDA in the cells treated with 0.1 μM D or with DA alone. These data indicate that PC12 cells treated with Aβ25-35 underwent peroxidation of their lipid bilayer, leading to increased formation of MDA, which was ameliorated by pretreatment with higher concentrations (1.0–10 μM) of D or DA.

**D and DA prevented Aβ-induced depletion of intracellular glutathione**

Glutathione (GSH, y-γ-glutamyl-L-cysteinyl-glycine), a low molecular weight thiol-containing molecule, is the most abundant intracellular antioxidant. It protects cells against reactive oxygen species (ROS)-induced oxidative stress and regulates intracellular redox status. Since D and DA significantly prevent glutamate-induced decreases in levels of GSH, the effects of D and DA on GSH levels in the Aβ-treated PC12 cells were examined. Aβ25-35 treatment (25 μM) for 24 h significantly reduced the intracellular GSH levels, to 35% of the levels observed for the untreated control cells. Preincubated PC12 cells with D (0.01–10 μM) or DA (1–10 μM) effectively prevented Aβ-induced depletion of intracellular GSH levels (Fig. 3). No changes in GSH levels were observed in the cells treated with D or with DA alone. These results indicate that D and DA have protective roles in maintaining cellular GSH levels, with differential effective concentrations between two compounds.

**D and DA prevented Aβ25-35-induced changes in the activity of antioxidant enzymes**

It has been suggested that superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and glutathione-S-transferase (GST) serve as the detoxifying system for the prevention of damage caused by ROS, and play pivotal roles in the scavenging of free radicals. To provide an insight into the relationship between the neuroprotective effect and the antioxidant effects of D and DA, the activity of these antioxidant enzymes was analyzed. As shown in Fig. 4A, 25 μM Aβ25-35 treatment resulted in an approximately 2.4-fold decrease in SOD activity as compared to the control.
contrast, PC12 cells exposed to the same concentration of Aβ25-35 in the presence of D (0.1–10 μM) and DA (0.01–10 μM) showed a markedly attenuated decrease in GPx activity. Treatment of PC12 cells with Aβ25-35 (25 μM) induced a significant decrease in CAT activity compared with the control group (p < 0.01) (Fig. 4C). Pretreatment with various concentrations of D (0.1–10 μM) and of Aβ25-35 (25 μM) significantly increased the activity of catalase, and this effect was strengthened with increasing D concentrations. When PC12 cells were exposed to D alone, CAT activity significantly increased, from 54.36 ± 6.31 nmol/min/mL (control) to 82.41 ± 0.30 nmol/min/mL (p < 0.01). CAT activity also markedly increased in the 10 μM DA exposure group as compared with the Aβ25-35 (25 μM) treated group. GST is a phase-II enzyme that is critically involved in the detoxification of xenobiotics as well as ROS. It catalyzes the conjugation of endogenous glutathione with a variety of electrophilic compounds, and expression of it is known to be modulated by Nrf2. 39–42) Aβ25-35 (25 μM) treatment for 24 h yielded a significant decrease in GST activity, to approximately 48% of the levels observed in the untreated control cells (Fig. 4D). A significant increase in the activity of GST was observed in the D and DA (0.1–10 μM) pretreatment groups. Taken together, these results indicate that pretreatment with D and with DA attenuated effectively the decreases in antioxidant enzyme activities caused by Aβ treatment.

D and DA increased the levels of nuclear Nrf2 in PC12 cells

Since transcription factor Nrf2 has been implicated as the central protein interacting with the antioxidant response element, also referred to as the electrophile response element, to induce a cluster of endogenous antioxidative enzymes, we investigated the possible involvement of D and DA in the levels of nuclear Nrf2 in PC12 cells. As shown in Fig. 5A and B, treatment with D and with DA (10 μM) produced a significant increase in Nrf2 protein levels within the nucleus, while Aβ25-35 (25 μM) treatment substantially decreased these levels. In addition, pre-incubation of PC12 cells with D and DA prior to Aβ25-35 treatment markedly enhanced nuclear Nrf2 levels with respect to Aβ25-35 alone.

D and DA inhibited aggregation of Aβ25-35

The effects of D and DA on Aβ aggregation were observed by ThT-induced fluorescence assay. ThT is known to associate rapidly with amloid aggregates, giving rise to a new emission maximum at about 482 nm. 43) As shown in Fig. 6A, when fresh Aβ25-35 (25 μM) was incubated at 37 °C, the fluorescence of ThT increased, following a characteristic sigmoidal curve. For Aβ alone, a lag time of 12 h was observed before aggregation started, as indicated by an increase in ThT fluorescence emission, which reached a peak at 72 h. The kinetics of Aβ aggregation was significantly lowered by 10 μM D and DA, as reflected in increases in the lag time to 24 and 72 h. This indicates that D and DA delay Aβ aggregation to a considerable extent. In order to determine whether D and DA are also inhibitory when added after initial Aβ aggregation events, their effects on the kinetics of disaggregation of preformed fibrils of Aβ25-35 were evaluated. Aβ preformed fibrils were mixed with 10 μM D and DA in two separate reaction mixtures and incubation was continued for another 48 h at 37 °C. ThT fluorescence intensity significantly decreased after the addition of D and DA (Fig. 6B). These results indicated that both D and DA inhibited Aβ aggregation and also destabilized preformed Aβ fibrils.

Discussion

Neurodegenerative diseases are a group of illnesses with diverse clinical implications and etiologies. There is a growing body of data implicating free radical toxicity, free radical-induced mutations, oxidative enzyme impairment, mitochondrial dysfunction, and excitotoxic mechanisms in the pathogenesis of neurodegeneration. 34–46) Oxidative stress caused by increased intracellular accumulation of ROS has been implicated in many pathophysiological processes, such as brain.
Neuroprotective Effects of Decursin and Decursinol Angelate

Fig. 6. Effects of D and DA on the Kinetics of Aggregation (A) and Disaggregation (B) of Aβ25-35. Reaction mixtures containing 25 μM Aβ25-35 (A) and 25 μM Aβ25-35 preincubated for 24 h at 37°C (B). 10μM D and DA were incubated at 37°C for the indicated times. The arrow in (B) indicates the time when D and DA were incubated in the reaction mixture. Data are expressed as fold of control values obtained in three experiments in triplicate.

ischemia, inflammation, and neurodegeneration, including AD.47–52) In recent years, numerous studies have suggested that the neurotoxicity of Aβ is one of the most important pathogenic factors in AD.53,54) The critical role of the C-terminal beta-sheet region combined with the S-oxidized radical cation of Met-35 in Aβ1-42 in the generation of free radicals, contributing to aggregation and stabilization of radicals, which in turn leads to long-lasting oxidative stress and neurotoxicity, was reported recently.55–57) There is also compelling evidence that oxidative stress plays a key role in the neuronal cell death induced by Aβ.58–61) The Aβ aggregation process is accelerated by transition metals that facilitate the oxidation of this neurotoxic peptide.62) It has been reported that Aβ produces H2O2, leading to hydroxyl radical formation via the Fenton reaction in the presence of transition metal ions.63) Aβ1-42 insult to neuronal cells has been identified as one of the major causes of AD, and Aβ25-35 has also been reported to be an active toxic fragment of Aβ1-42.64,65) Furthermore, Aβ25-35 and Aβ1-42 have been found to induce similar effects in neuritic atrophy and cell death.66) Therefore, in this study, Aβ25-35 was employed as a neurotoxicant. One feasible approach to prevent free radical-mediated cellular injury is to augment or potentiate oxidative defense capacity through dietary or pharmacological intake of antioxidants. Moreover, induction of endogenous detoxifying enzymes and of antioxidative proteins appears to be an effective strategy to delay disease progression and the toxic effects associated with Aβ-mediated cytotoxicity.57–70)

D and DA, the two major coumarins isolated from Angelica gigas, have been reported to possess many physiological functions, including anti-cancer,18) anti-bacterial,23) anti-nematodal,25) and antioxidant.26,71,72) A previous study also found that D and DA have anti-amnesic effects against Aβ.73) The majority of clinical studies on the use of antioxidants as neuroprotective agents have had very limited success, primarily due to the impermeability of the blood-brain-barrier (BBB) to most of the compounds investigated. However, the pioneering work of Madgula et al. identified that D and DA have the potential to cross the BBB and to penetrate the CNS.74) These observations support the use of D and DA in the treatment of CNS disorders including AD. In the present study, we found for the first time that the D- and DA-induced Nrf2-associated ARE response occurs readily and constitutes a protective strategy against the oxidative action of Aβ in PC12 cells. Treatment with low doses of D and DA (0.01–10μM) protected PC12 cells against Aβ-induced cytotoxicity (Fig. 1B), whereas treatment with a high dose of D and DA (50μM) increased cytotoxicity (Fig. 1A). These results indicate that D and DA play dual roles in cell death based on their concentrations.

Lipid peroxidation is one of the major outcomes of free radical-mediated injury, which directly damages membranes and generates a number of secondary products from fission and endo-cyclization of oxygenated fatty acids that possess neurotoxic activities. MDA, an end product of membrane lipid peroxidation, is one of the most widely used markers of free radical-mediated damage,76) and increased MDA levels have been observed in patients with neurodegenerative diseases.76) In the present study, MDA levels were markedly increased in the cells treated with Aβ25-35, and D and DA treatment decreased the levels of MDA as compared with the control cells. This indicates that D and DA have a preventive effect against Aβ-mediated lipid peroxidation in PC12 cells.

GSH is an important intracellular antioxidant and an essential cofactor for antioxidant enzymes that protect against endogenous oxygen radicals. Glutathione disulfide (GSSG) is referred to as an oxidized GSH, and disturbance of GSH homeostasis can lead to or result from oxidative stress in neurodegenerative disorders.77) Increasing evidence indicates that GSH plays an important role in the detoxification of ROS in the brain.78) Pretreatment with a GSH precursor, N-acetyl-L-cysteine, prevented Aβ-induced cytotoxicity and peroxide accumulation.79) The elevated ratio of GSSG to GSH in Parkinson’s disease (PD) is consistent with the concept of oxidative stress as an important component in the pathogenesis of PD.80) A lowered GSH content appears to be the first indicator of oxidative stress during the progression of PD and AD.81,82) It has been reported that oxidative stress plays a role in the pathogenic process, and that alterations in the GSH system are secondary to other events leading to neurodegeneration. Thus it remains debatable whether this is a primary defect or only a consequence of ROS generation. Nevertheless, D and DA treatment can lead to the enhanced synthesis of GSH or inhibition of its degra-
dation, resulting in a slowing of Aβ-induced autophagic signaling. The increase in GSH levels upon D and DA treatment should result in a clinical benefit and/or neuro-protection in Aβ-associated diseases.

Endogenous antioxidant enzymes represent the first line of defense of the brain in countering the deleterious effects of ROS. Generally, the cytoprotective properties of antioxidants are partially attributed to their ability to induce antioxidant enzymes. The cytoprotective mechanism of D and DA against Aβ-mediated oxidative stress was also investigated by assessing the status of the antioxidant enzymes SOD, GPx, CAT, and GST. SOD, the first line of defense against free radicals, was found to have ROS-metabolizing activity. CAT is also a major primary antioxidant defense component, having a function similar to GPx. In the present study, augmented activity of GPx and CAT was observed in PC12 cells exposed to D and DA before Aβ25-35 treatment, and this may have been responsible for the increased resistance to oxidative stress. Another finding of this study is that D and DA significantly upregulated the GST activity level following Aβ25-35 challenge in PC12 cells, since GST is a secondary antioxidant enzyme that plays an important role in detoxifying ROS by maintaining a ready supply of intermediates such as GSH and nicotinamide adenine dinucleotide phosphate (NADPH). The preliminary in vitro data given herein appear to bear out the postulate that the cytoprotective effects of D and DA are related to the increased activity of antioxidant enzymes.

Recently, Nrf2 has been found to be a critical transcription factor that binds to the ARE in the promoter region of a number of target genes encoding for antioxidant enzymes in several types of cells and tissues. Nrf2 regulates the expression of many detoxifying genes, including SOD, CAT, GPx, and GST. The proteins products of these genes provide multiple layers of protection during cellular insult, and collectively favor cell survival. The ability of Nrf2 to upregulate the expression of antioxidant enzymes via ARE suggests that increasing the Nrf2 level may provide a useful system for combating oxidative insults. Nrf2 knockout mice (Nrf2-/-) possess much lower levels of phase II detoxifying enzymes and are more susceptible to oxidative stress and carcinogen-induced tumorigenesis than wild-type animals. Various reports also have underscored the cytoprotective effects of Nrf2-induced antioxidant protection in distinct CNS cell types and animal models of neurodegeneration. Nrf2-driven antioxidant enzymes protect primary astrocytes from H2O2-induced apoptosis, and decreased levels of it result in increased susceptibility to oxidative stress, whereas overexpression of Nrf2 protects neurons from oxidative stress. Beneficial effects of dietary tert-butylhydroquinone and sulforaphane, both potent inducers of the Nrf2-ARE pathway, have been reported in animal models of neurodegeneration and cerebral ischemia. Although several chemopreventive compounds have been found to induce Nrf2, antioxidant and detoxification genes, the effects of D and DA on Nrf2 activation during oxidative damage of PC12 cells have not been considered prior to this study. To determine whether D and DA would activate Nrf2 in PC12 cells using Western blotting. As shown in our results, the levels of Nrf2 were markedly increased in the nuclei of PC12 cells exposed to a non-toxic concentration of D and DA (Fig. 5). The increased levels of nuclear Nrf2 following D and DA treatment were associated with a marked increase in antioxidative enzyme activity or antioxidant content. These data suggest that antioxidant defense is enhanced by D and DA via activation of Nrf2 and upregulation of antioxidative enzyme activity.

One of the current therapeutic approaches in AD is to prevent Aβ fibrillation and to destabilize preformed fibrils using small biomolecules. Various biomolecules, along with their ability to attenuate oxidative stress, have also been reported to possess anti-amyloidogenic effects in AD. The present study on the anti-aggregation of D and DA indicates an inhibitory role of D and DA in Aβ aggregate formation in the AD brain. D and DA might interact with the peptide side chain to inactivate fibril aggregation. Further research is needed to elucidate the exact mechanism by which D and DA distort Aβ fibrils.

Taken together, these results suggest that D and DA stimulate the activation of Nrf2 and thereby elevate the activity of antioxidant enzymes that contribute to the defense mechanism against Aβ25-35-induced oxidative injury. In addition, the protective effect of D and DA may arise from anti-Aβ aggregation. These findings indicate that the use of purified D and DA should be considered as an adjunct therapeutic strategy to combat neural demise in AD and other oxidative stress-related diseases. Further in vivo study with D and DA should substantiate this therapeutic potential of these compounds.

References