The Neuroprotective Effects of Phytoestrogens on Amyloid β Protein-induced Toxicity Are Mediated by Abrogating the Activation of Caspase Cascade in Rat Cortical Neurons*

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Amyloid β protein (Aβ) elicits a toxic effect on neurons in vitro and in vivo. In present study we attempt to elucidate the mechanism by which Aβ confers its neurotoxicity. The neuroprotective effects of phytoestrogens on Aβ-mediated toxicity were also investigated. Cortical neurons treated with 5 μM Aβ-(25–35) for 40 h decreased the cell viability by 45.5 ± 4.8% concomitant with the appearance of apoptotic morphology. 50 μM kaempferol and apigenin decreased the Aβ-induced cell death by 81.5 ± 9.4% and 49.2 ± 9.9%, respectively. Aβ increased the activity of caspase 3 by 10.6-fold and to a lesser extent for caspase 2, 8, and 9. The Aβ-induced activation of caspase 3 and release of cytochrome c showed a bi-phasic pattern. Apigenin abrogated Aβ-induced cytochrome c release, and the activation of caspase cascade. Kaempferol showed a similar effect but to a lesser extent. Kaempferol was also capable of eliminating Aβ-induced accumulation of reactive oxygen species. These two events accounted for the remarkable effect of kaempferol on neuroprotection. Quercetin and probo- cul did not affect the Aβ-mediated neurotoxicity. However, they potentiated the protective effect of apigenin. Therefore, these results demonstrate that Aβ elicits activation of caspase cascades and reactive oxygen species accumulation, thereby causing neuronal death. The blockade of caspase activation conferred the major neuroprotective effect of phytoestrogens. The antioxidative activity of phytoestrogens also modulated their neuroprotective effects on Aβ-mediated toxicity.

Senile plaque and neurofilament tangles are the hallmarks of Alzheimer’s disease (AD), which is one of the major neuro-degenerative diseases. Amyloid β protein (Aβ), the major protein component of senile plaque, has been suggested to play an important role in pathogenesis of AD (1, 2). Aβ, a 39- to 42-amino acid peptide, is derived proteolytically from amyloid precursor protein, which is expressed widely throughout the brain (3, 4). Multiple lines of evidence have demonstrated that fibril Aβ participates in the induction of neuronal death and neuritic changes (5–8). The Aβ-related fragments (Aβ-(1–40), Aβ-(1–42), and Aβ-(25–35)) exhibit toxicity to neurons either in vitro or in vivo (9–11). The mechanisms by which Aβ elicits deleterious effect on neurons remain to be established.

Extensive studies have shown that Aβ-induced neurotoxicity in multiple cell types may be mediated by several different mechanisms. The neurotoxic effect may be attributable to the disturbance in calcium homeostasis (12–14) and consequently inducing the accumulation of reactive oxygen species (ROS) (13, 15–18). ROS provokes membrane damage compromising membrane integrity and increasing the permeability of ions, including calcium. The increase of calcium influx leads to generating more ROS, thereby initiating the positive feedback loop. Cultured neurons treated with Aβ or transgenic mice expressing Aβ renders neurons vulnerable to apoptosis, indicating that caspase activation plays a role in Aβ-induced neurotoxicity. Several caspases involved in apoptosis have been described to be activated by Aβ (6, 19–22). However, the mechanisms by which Aβ activates caspase cascade remain unclear. Furthermore, the activation of cyclin-dependent kinase has also been indicated to be a mediator of neurotoxicity induced by Aβ (23).

Several agents have been shown to be neuroprotective in in vitro system by targeting to specific pathway responsible for Aβ-induced toxicity. These agents include antioxidants or free radical scavengers (12, 15, 17, 18, 24–26), calcium ion channel blockers (13, 26); growth factors (27), inhibitor of cyclin-dependent kinase (23), and caspase inhibitors (15, 19, 21). Recent evidence shows that estrogen deficiency in postmenopausal women is one of the most significant risk factors for onset of AD (28, 29). Thus, estrogens have become a research focus. It has been shown that estrogen protects neurons against a number of toxic insults, including Aβ (30–34). The neuroprotective effects of estrogen are suggested to be independent of their classic nuclear estrogen receptors (32, 34).

Flavonoids, the so-called phytoestrogens, occur ubiquitously in food plants and herbal medicines. They not only bind to estrogen receptor but also exert antioxidative and antiproliferative activity (35–37). Furthermore, evidence has demonstrated that phytoestrogens interfere with a number of intracellular processes, including enzyme activation and cAMP accumulation (38, 39). Flavonoids are typically classified into four groups. Those are flavone, flavonol, flavanone, and isofla-
vone. Thus, apigenin, luteolin, kaempferol, quercetin, narigenin, 2,3-dihydroluteolin, genistein, and prunetin were chosen for the study of neuroprotection (Fig. 1).

In the present report, we demonstrate that apigenin blocked the release of cytochrome c and activation of caspase cascade induced by Aβ. Kaempferol only inhibited the activation of caspase cascade, and the effect was less potent than that of apigenin. However, kaempferol was more effective than apigenin in counteract the deleterious effect of Aβ on cortical neurons. Kaempferol exhibited antioxidative activity and decreased the ROS accumulation induced by Aβ, whereas apigenin lacked antioxidative activity and showed a marginal effect on ROS level. Furthermore, quercetin or probucol facilitated the neuroprotective effect of apigenin on Aβ-mediated toxicity. Therefore, these results indicated that blockade of activation of caspase cascade conferred the neuroprotective effects of phytoestrogens on Aβ-mediated neurotoxicity. The inhibition of caspase cascade in combination with antioxidative activity will further eliminate Aβ-mediated neurotoxicity.

EXPERIMENTAL PROCEDURES

Materials

Media and supplements for cell culture were from Life Technologies (Gaithersburg, MD). Amyloid β peptide-(25–35) (Aβ-(25–35)), poly-l-lysine, cytosine-β-D-arabinofuranoside, probucol, genistein, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), trypan blue, dimethyl sulfoxide (Me2SO), and fluorescein diacetate were purchased from Sigma Chemical Co. (St. Louis, MO). Enhanced chemiluminescence detection reagents and anti-mouse IgG antibody conjugated with horseradish peroxidase were obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Fig. 1. The structures and chemical names of phytoestrogens.

| Trivial name | Chemical name |
|--------------|---------------|
| **Flavones:** |               |
| Apigenin     | 4',5,7-trihydroxyflavone |
| Luteolin     | 3',4',5,7-tetrahydroxyflavone |
| **Flavonols:** |               |
| Kaempferol   | 3',4',5,7-tetrahydroxyflavone |
| Quercetin    | 3,3',4',5,7-pentahydroxyflavone |
| **Flavanones:** |               |
| Narigenin    | 4',5,7-trihydroxyflavone |
| 2,3-dihydroluteolin | 3',4',5,7-tetrahydroxyflavone |
| **Isoflavones:** |               |
| Genistein    | 4',5,7-trihydroxyisoflavone |
| Prunetin     | 4',5-dihydroxy-7-methoxyisoflavone |

Fig. 2. Effects of Aβ-(25–35) on the cell viability of cortical neurons. Cortical neurons were incubated with various concentrations of Aβ-(25–35) for 40 h (A), and neurotoxicity was measured by the FDA assay (closed circles), MTT assay (closed squares), trypan blue exclusion (open squares), and LDH release (closed triangles). Neurons were exposed to 5 μM of Aβ-(25–35) for 0–40 h, after the incubation periods, and cell viability was determined by the MTT assay (B). Results are means ± S.D. (where large enough to be shown) from four independent experiments and expressed relative to the cells treated with vehicle alone. C, the representative photographs of neurons treated with 0 (a), 5 (b), 10 (c), and 20 μM (d) of Aβ-(25–35) for 24 h.
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Cortical neurons were incubated with vehicle (0.1% Me₂SO), various phytoestrogens, or β-estradiol at highest nontoxic concentration for 2 h then exposed to 5 μM Aβ for 40 h. The cell viability was assessed by MTT reduction analysis. The data are means ± S.D. and expressed relative to cells treated with vehicle alone. Significant differences between cells treated with Aβ and Aβ plus phytoestrogen or estrogen are indicated in footnotes.

| Cell death | % |
|------------|---|
| 5 μM Aβ (0.1% Me₂SO) | 45.5 ± 4.6 |
| 5 μM Aβ plus Reagent (μM) | |
| Flavones | |
| Apigenin (50) | 25.0 ± 5.9a |
| Luteolin (50) | 42.7 ± 3.1 |
| Flavonols | |
| Kaempferol (50) | 7.9 ± 3.6a |
| Quercetin (50) | 41.2 ± 2.1 |
| Flavanones | |
| Naringenin (50) | 41.6 ± 5.3 |
| 2,3-Dihydroquercetin (20) | 58.3 ± 8.2 |
| Isoflavones | |
| Genistein (30) | 50.1 ± 4.8 |
| Prunetin (10) | 45.0 ± 5.0 |
| Estrogen | |
| β-Estradiol (10) | 54.0 ± 3.7 |

a p < 0.001.

Biotech (Buckinghamshire, UK). 2,2’-Azinobis(3-ethylbenzthiazoline sulfonic acid) was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Fluorescein diacetate (FDA) and 5-(and-6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) were purchased from Molecular Probes (Leiden, The Netherlands). Ac-DEVD-CHO, Ac-LEHD-CHO, Ac-IETD-CHO, and a caspase-3 cellular activity assay kit were obtained from Calbiochem (Darmstadt, Germany). Monoclonal antibodies for cytochrome c were obtained from PharMingen (San Diago, CA). All other reagents were purchased from Sigma or Merck (Darmstadt, Germany).

**Methods**

**Cell Culture**—Primary cultures of neonatal cortical neurons were prepared from the cerebral cortex of Harlan Sprague-Dawley rat pups at postnatal day 1 (40, 41). Briefly, each pup was decapitated and the cortex was digested in 0.5 mg/ml papain at 37 °C for 15 min. The tissue was dissociated in Hypermate A medium (containing B27 supplement) by aspirating trituration. Cells were plated (5 × 10⁶ cells/cm²) onto poly-D-lysine-coated dishes and maintained in Neurobasal medium containing B27 supplement (40), 10 units/ml penicillin, 10 μg/ml streptomycin, and 0.5 μg/ml glutamine (5% CO₂/9% O₂) for 3 days. Cells were then exposed to cytoxic β-β-arabinofuranoside (5 μM) for 1 day to inhibit proliferation of non-neuronal cells. The cells were used for the experiment on the fifth day.

**Measurement of Cell Viability**—The reduction of MTT, cleavage of MTC, and trypan blue exclusion were used to evaluate the cell viability. Cells were incubated with minimum essential medium containing 0.5 mg/ml MTT for 1 h. The medium was aspirated, and the formazan particle was dissolved with lysis buffer (10% sodium dodecyl sulfate, 3.3 mM HCl, 50 mM dimethylformamide). A₆₃₅nm was measured by using enzyme-linked immunosorbent assay reader (42). Cells were loaded with 15 μM FDA for 5 min at 25 °C, and then 1 ml of 1% deoxycholate was added to lyse the cells. The fluorescent intensity of the lystate was determined by using a spectrofluorometer with excitation and emission wavelength of 490 nm and 514 nm, respectively (26, 40). Cell viability was also assessed by using trypan blue exclusion as described previously (42).

**Measurement of ROS**—Intracellular reactive oxygen species were measured by CM-H₂DCFDA assay (26). In brief, cells were loaded with 50 μM CM-H₂DCFDA for 30 min, and then 1 ml of 1% deoxycholate was added to lyse the cells. The fluorescent intensity of the lystate was determined by using a spectrofluorometer with excitation and emission wavelength of 490 nm and 514 nm, respectively (26, 40).
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Results

Phytoestrogens Protected Neurons from Aβ-induced Neurotoxicity—Treatment of rat cortical neurons with the toxic fragment of fibril Aβ (f-Aβ-(25–35)) for 40 h decreased cell viability in a concentration-dependent manner as determined by MTT reduction or trypan blue exclusion (Fig. 2A). 10 μM Aβ decreased the MTT reduction and trypan blue exclusion by 58.2 ± 3.9% and 63.3 ± 11.5%, respectively. However, treatment with Aβ elicited a marginal effect on the ability to cleave fluorescein diacetate and had no effect on the release of LDH. The effect of Aβ on cell viability of cortical neurons was time-dependent as measured by MTT reduction (Fig. 2B). The appearance of irregularly shaped cell bodies and discontinuous neurite was concomitant with the decrease of cell viability (Fig. 2C). The percentage of cells with injured morphology was elevated as the concentration of Aβ was increased.

The effects of a series of phytoestrogens on Aβ-induced neurotoxicity were investigated. Cells were incubated with various concentrations of phytoestrogens for 2 h then exposed to 5 μM Aβ for 40 h. Cell viability was verified by MTT reduction analysis. Results showed that kaempferol and apigenin reduced the Aβ-induced neurotoxicity in a concentration-dependent manner (Fig. 3, A and B). Kaempferol at 50 μM decreased the percentage of cell death from 45.5 ± 4.6% to 7.9 ± 3.8%. Apigenin was less potent to attenuate Aβ-induced neurotoxicity (Fig. 3B). For the morphology, 50 μM kaempferol diminished the extent of cells with injured morphology induced by Aβ (Fig. 3C). The protective effect of kaempferol on Aβ-mediated neurotoxicity was further confirmed by the measurement of trypan blue exclusion (data not shown). Luteolin, quercetin, flavonones, isoflavonones, and β-estradiol did not show any significant effects on Aβ-induced neurotoxicity (Table 1).

Aβ Induced the Activation of Caspase Cascade—Inhibitors of caspases were employed to investigate whether apoptosis was involved in Aβ-mediated toxicity. Ac-DEVD-CHO and Ac-LEHD-CHO are the cell-permeable inhibitors for caspases 3 and 9, respectively.
and 9, respectively. These inhibitors block the activity of caspases but do not interfere with its activation. Both inhibitors reduced Aβ-induced cell death in a concentration-dependent manner (Fig. 4, A and B). In contrary, Ac-IETD-CHO, the cell-permeable inhibitor of caspase 8, did not decrease Aβ-induced cell death (data not shown). Morphological study also showed that Ac-DEVD-CHO and Ac-LEHD-CHO eliminated the cells with injured morphology induced by Aβ (Fig. 4C).

Analysis of the activity of caspases were performed to further determine whether the activation of caspase cascade was involved in the Aβ-mediated neurotoxicity. Treatment of neurons with Aβ induced activation of caspase 2, 3, 8, and 9 in a time-dependent manner as measured by substrate cleavage (Fig. 5). Exposure of neurons to Aβ for 24 h, the specific activity of caspase 2, 3, 8, and 9 were increased by 6.4-, 11.6-, 6.0-, and 4.7-fold of control, respectively. The activity of caspase 3 was enhanced to a larger extent by Aβ in comparison with caspase 2, 8, and 9. Aβ exhibited a biphasic effect on the activation of caspase 3. There was a transit activation of caspase 3 at 2 h and followed by a sustained activation from 8 to 24 h. The similar result for caspase 9 was also obtained. However, the first wave of activation of caspase 9 at 2 h was not statistically significant. Caspases 2 and 8 did not show early phase of activation. The significant activation of caspases 2 and 8 occurred from 12 to 24 h.

The Effects of Caspase Inhibitors and Phytoestrogens on Caspase Cascade—The inhibitor of caspases 3, 9, and 8 decreased the activity of caspase 3 and 9 at 2 h (Table II). Both inhibitors of caspase 3 and 9 inhibited the activity of caspase 2, 3, and 9 at 24 h (Table III). The inhibitor of caspase 3 diminished the activity of caspase 2, 3, and 9 by 72.8 ± 6.8, 90.8 ± 4.4, and 60.9 ± 15.0%, respectively, and the inhibitor of caspase 9 decreased the activity of caspase to a lesser extent. However, both inhibitors did not show significant effect on the activity of caspase 8. The inhibitor of caspase 8 decreased the activity of caspase 8 and 9 by 17.0 and ± 4.0% and 43.4 ± 4.0%, respectively.

Apigenin and kaempferol did not show significant effects on the activation of caspase cascade at 2 h (Table II). However, apigenin inhibited the activity of caspase 2, 3, 8, and 9 at 24 h indicated time periods. Activity of caspase 9, 3, 2, and 8 was determined by the cleavage ability of Ac-LEHD-pNA (a), Ac-DEVD-pNA (b), Ac-VDVAD-pNA (c), and Ac-IETD-pNA (d) in cell extracts, respectively. Results are means ± S.D. (where large enough to be shown) from four independent experiments. Significant differences between control and Aβ-treated cells are indicated by *, p < 0.05 and ***, p < 0.001.

### Table II

| Reagents                  | Caspase 3 | Caspase 9 |
|---------------------------|-----------|-----------|
| Apigenin                 | 100.6 ± 16.8 | 100.3 ± 28.6 |
| Kaempferol               | 112.5 ± 15.2 | 84.3 ± 34.9 |
| Quercetin                | 113.3 ± 10.3* | 87.2 ± 19.3 |
| Ac-DEVD-CHO (caspase 3 inhibitor) | 2.9 ± 3.7b  | 25.9 ± 6.9b  |
| Ac-LEHD-CHO (caspase 9 inhibitor) | 76.7 ± 8.4b  | 82.7 ± 17.2b  |
| Ac-IETD-CHO (caspase 8 inhibitor) | 60.8 ± 8.0b  | 31.7 ± 12.7b |

* p < 0.01.  
** p < 0.001.
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The inhibition of caspase activation at 24 h by either phytoestrogen or caspase inhibitors

Cortical neurons were incubated with phytoestrogens (50 μM) or caspase inhibitor (10 μM) for 2 h and exposed to 5 μM Aβ for 24 h. Cell viability was assessed by MTT reduction analysis. The data are means ± S.D. of four independent experiments and expressed relative to cell treated with Aβ alone. Significant differences between cells treated with Aβ and Aβ plus phytoestrogen or caspase inhibitor are indicated in footnotes.

| Reagents                        | Caspase 2 | Caspase 3 | Caspase 8 | Caspase 9 |
|--------------------------------|-----------|-----------|-----------|-----------|
| Apigenin                       | 40.8 ± 11.2b | 44.5 ± 12.3b | 36.8 ± 18.0b | 46.2 ± 18.0b |
| Kaempferol                     | 56.7 ± 8.2b  | 67.7 ± 6.9b  | 47.2 ± 14.1b | 51.7 ± 14.1b |
| Quercetin                      | 104.5 ± 13.5 | 92.6 ± 17.1 | 112.0 ± 8.3b | 79.8 ± 6.4b  |
| Ac-DEVD-CHO (caspase 3 inhibitor) | 27.2 ± 8.5b  | 9.3 ± 4.5b   | 97.0 ± 13.8 | 39.1 ± 15.0b |
| Ac-LEDH-CHO (caspase 9 inhibitor) | 71.2 ± 10.9b | 59.4 ± 10.1b | 93.4 ± 11.1 | 48.9 ± 19.3b |
| Ac-IETD-CHO (caspase 8 inhibitor) | 94.4 ± 7.1   | 95.4 ± 9.1   | 83.0 ± 4.0b  | 56.6 ± 4.0b  |

* p < 0.01.
* b p < 0.001.

DISCUSSION

In the present report, we demonstrate that kaempferol and apigenin prevented death of cultured neurons exposed to fibril Aβ(25–35). The action modes of these phytoestrogens were neither due to an activation of the nuclear estrogen receptor nor solely based on an antioxidative mechanism. The anti-apoptotic signaling activity conferred neuroprotective effect of kaempferol and apigenin. Antioxidative activity of flavonoids or other antioxidants did not exhibit direct effects on neuroprotection. However, antioxidative activity facilitated the neuroprotective effect of apigenin.

Kaempferol and apigenin protected neurons from Aβ-mediated toxicity, whereas quercetin and luteolin failed to protect neurons. This result suggests that the substitution of hydroxyl group at C-3’ position severely impairs the neuroprotective ability of kaempferol. The deficiency of neuroprotective effects of narigenin and 2,3-dihydroluteolin also implicates that the antioxidative activity of flavonoid and other antioxidants did not exhibit direct effects on neuroprotection.

Antioxidative Activity Potentiated the Neuroprotection of Apigenin—The effect of Aβ on intracellular level of ROS was examined by using 5-(and-6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate (CM-H2DCFDA). Aβ induced ROS accumulation significantly from 8 to 24 h (Fig. 7A). Treatment with 5 μM Aβ for 8 and 16 h elevated the level of ROS to 129 ± 5% and 158 ± 10% of control, respectively. Aβ increased the level of ROS in a concentration-dependent manner (Fig. 7B). Kaempferol, luteolin, and quercetin reduced the level of ROS by 30–50% in control cells and by 50–60% in Aβ-treated cells (Fig. 7C). Apigenin decreased the ROS level by 25% in Aβ-treated cells but did not affect that in control cells. The antioxidant capacity of phytoestrogens was also determined by TEAC method (Table IV). The results showed that apigenin had lower antioxidant capacity than quercetin and luteolin. Kaempferol also showed higher antioxidant capacity than apigenin. Thus, the sequence of antioxidant capacity for these phytoestrogens did not correlate well to their neuroprotective ability. The results also implied that high antioxidative activity of flavonoid per se was not able to protect neuron against Aβ-induced neurotoxicity.

Apigenin was much more able than kaempferol to block caspase activation (Table III). Apigenin, however, exhibited the neuroprotective effect to a lesser extent (Fig. 5). We speculated, therefore, that the antioxidative activity and ability for ROS reducing may modulate the neuroprotective effect of kaempferol. To address the hypothesis that antioxidative activity may enhance the neuroprotective effect of apigenin, the effect of antioxidants on neuroprotection of apigenin was evaluated (Fig. 5). Quercetin did not reduce Aβ-mediated cell death from 1 to 50 μM. Cotreatment with quercetin and apigenin enhanced the neuroprotective effect of apigenin (Fig. 8A). Probucol, an antioxidant, was more potent at potentiating the neuroprotection effect of apigenin (Fig. 8B). The results demonstrated that, although antioxidant activity of quercetin and probucol per se did not show neuroprotective effect, they did modulate the neuroprotective effect of apigenin.

Phytoestrogens reducing the loss of MTT reduction by Aβ has also been described in PC12 phenoehromocytoma cells and human neuroblastoma cells (32, 33, 45, 46). A possible explanation of these discrepancies could be Aβ exerting toxic effect on cell line and primary culture of cortical neurons via distinct mechanisms. Phytoestrogens reducing the antioxidative activity of flavonoid per se was not able to protect neuron against Aβ-induced neurotoxicity.

by about 55 to 60% (Table III). Kaempferol also diminished the activity of these caspases but to a lesser extent. Quercetin only showed inhibitory effect on the activity of caspase 9 at 24 h (Table III).

**Apigenin Inhibited Aβ-induced Release of Cytochrome c—**Kaempferol and apigenin attenuated Aβ-induced activation of caspases 9 and 3. The release of cytochrome c from mitochondria, the upstream signaling component of these two caspases, was therefore investigated (Fig. 6). The release of cytochrome c induced by Aβ exhibited a biphasic pattern similar to the activation of caspase 3. The first phase occurred at 2 h (Fig. 6A) and followed by the second phase from 12 to 24 h (data not shown). Apigenin significantly inhibited Aβ-induced cytochrome c release at 2 and 12 h by 34.1 and 55.7%, respectively. However, kaempferol did not affect Aβ-induced cytochrome c release either at 2 or 12 h.

**Antioxidative Activity Potentiated the Neuroprotection of Apigenin—**The effect of Aβ on intracellular level of ROS was examined by using 5-(and-6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate (CM-H2DCFDA). Aβ induced ROS accumulation significantly from 8 to 24 h (Fig. 7A). Treatment with 5 μM Aβ for 8 and 16 h elevated the level of ROS to 129 ± 5% and 158 ± 10% of control, respectively. Aβ increased the level of ROS in a concentration-dependent manner (Fig. 7B). Kaempferol, luteolin, and quercetin reduced the level of ROS by 30–50% in control cells and by 50–60% in Aβ-treated cells (Fig. 7C). Apigenin decreased the level of Aβ in 25% in Aβ-treated cells but did not affect that in control cells. The antioxidant capacity of phytoestrogens was also determined by TEAC method (Table IV). The results showed that apigenin had lower antioxidant capacity than quercetin and luteolin. Kaempferol also showed higher antioxidant capacity than apigenin. Thus, the sequence of antioxidant capacity for these phytoestrogens did not correlate well to their neuroprotective ability. The results also implied that high antioxidative activity of flavonoid per se was not able to protect neuron against Aβ-induced neurotoxicity.

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sustained activation after 8 h. For caspase 9, the first wave activation was not supported by the statistics. Nevertheless, the elevation of caspase 9 activity at 2 h may be biologically relevant. The release of cytochrome c also showed a similar pattern as the activation of caspase 3 and 9, although Aβ activated caspase 2 and 8 after 16 h and to a lesser extent. These data suggest that the apoptosis signaling induced by Aβ is mediated primarily by activation of caspase 3 and 9 and...
The activation of caspase 2 and 8 and the production of ROS may be the secondary responses. Caspase 8 inhibitor was found unable to prevent neuronal death induced by Aβ, which further confirms that caspase 8 may not play the major role in Aβ-induced neuronal death.

Although the Aβ-mediated neurotoxicity becomes the focus of intense interest, the underlining mechanisms are still controversial. The study of Giovanni et al. shows that cortical neurons from caspase 3 knockout mice are resistant to Aβ-mediated cell death, suggesting that caspase 3 is the major component mediating Aβ-induced apoptosis (22). However, the studies of Troy et al. (21) show down-regulation of caspase 3 does not block Aβ-(1–42)-induced cell death. They also show that sympathetic neurons from caspase 2 null mice are resistant to Aβ-(1–42)-mediated cell death, implicating an important role of caspase 2 in Aβ-(1–42)-induced apoptosis (21). Moreover, cell death is blocked by the down-regulation of caspase 2 in hippocampal neurons, sympathetic neurons, or neuronal PC12 cells with antisense oligonucleotides. Beside caspase 3 and 2, other caspases are also thought to be involved in Aβ-induced apoptosis. Nakagawa et al. (20) shows that caspase-12-deficient cortical neurons are not susceptible to the apoptosis induced by Aβ-(1–40) (20). Another study, however, shows that the apoptotic pathway activated by Aβ requires both caspase 8 and Fas-associated death domain (FADD) (47). Our results demonstrate that Aβ elicited the activation of caspase 2, 3, 8, and 9 in cortical neurons. The release of cytochrome c and activation of caspase 9 and caspase 3 may be the major pathway mediating the Aβ-induced apoptosis of neurons. Kaempferol and apigenin abrogated the activation of all four caspases (Table III). Apigenin was more potent than kaempferol in blocking the release of cytochrome c and activation of caspase cascade induced by Aβ. However, kaempferol was more effective than apigenin to protect neurons from Aβ-induced cell death. On the basis of these data, we speculate that there may be other factors involved in the protective effect of kaempferol. ROS scavenging activity of kaempferol may be the most possible candidate to promote its neuroprotective effect.

Many reports have demonstrated or proposed that ROS is responsible for Aβ-induced neurotoxicity (13, 15–18). Behl et al. (17) shows that Aβ increases the intracellular level of H₂O₂ and...
lipid peroxide. This result suggests that free radical damage is one factor accounting for Aβ cytotoxicity. The studies of Kruman et al. (48) further provide evidence that 4-hydroxynonenal, an aldehydic product of membrane lipid peroxidation, is a key mediator of neuronal apoptosis induced by Aβ. Therefore, it is possible that scavenging of ROS may also contribute to the neuroprotective activity of kaempferol and apigenin. The level of ROS in Aβ-treated neurons was elevated after 8-h incubation, implying that ROS production may be another mediator for Aβ-induced cell death. The level of ROS in Aβ-treated neurons were reduced by both neuroprotective and nonprotective flavonoids. Furthermore, nonprotective flavonoids, quercetin and luteolin, were more potent in reducing the level of ROS and had higher antioxidative activity. These results indicate that the antioxidative activity of flavonoids per se do not confer the neuroprotective effect on Aβ-mediated toxicity. Nevertheless, quercetin and p-hydroxybenzaldehyde, which possess antioxidative activity, did show a significant facilitating effect on the neuroprotection of apigenin. These data provide the convincing explanation for the inconsistent results between inhibition of caspase cascade and the neuroprotective effect of kaempferol and apigenin.

Taken together, the results presented here provide a plausible mechanism by which Aβ provokes death of cortical neurons. In this model (see Fig. 9), Aβ-mediated apoptosis consists of the first and second waves of caspases activation. Aβ primarily induces the release of cytochrome c from mitochondria and subsequent activation of caspase 9 and 3 at 2 h (Figs. 5 and 6). The inhibitor of caspase 8 blocked the activation of caspase 9 and 3 at 2 h (Table II), indicating caspase 8 may be involved in the first wave of caspase activation. Thereafter, caspase 3 evokes a second wave of cytochrome c release and activation of caspase cascade from 12 to 24 h. In the second wave of caspase activation, there is a positive feedback of caspase 3 and cytochrome c release, thereby establishing a signaling cascade and amplifying the signal (49). The inhibitor of caspase 3 abrogated the activation of caspase 2 at 24 h (Table III), indicating that caspase 2 may be the downstream factor of caspase 3 and compensated in the second wave of caspase activation. The sustained activation of caspase 8 occurred after 12 h, indicating that caspase 8 may also participate in the second wave of caspase activation. Furthermore, the damaged mitochondria in the second wave of caspase activation may also cause the accumulation of ROS, or alternatively, the generation of ROS after 8 h may also be involved in the release of cytochrome c and the second wave activation of caspase cascade. Both activation of caspase cascade and elevation of ROS may account for the Aβ-mediated toxicity. Both apigenin and kaempferol, but not quercetin, abrogated the second wave of cytochrome c release or activation of caspase 2, 3, 8, and 9 (Table III, Fig. 6). Despite apigenin abrogating the initial wave of cytochrome c release, both flavonoids did not affect the initial wave activation of caspase 3 and 9. These results suggest that apigenin and kaempferol protect neurons against Aβ-induced toxicity by diminishing the second wave activation of the caspase cascade. Our results clearly demonstrate that kaempferol and apigenin exhibited differentially neuroprotective effects on Aβ-induced toxicity by abrogating the release of cytochrome c and activation of the caspase cascade. Antioxidative activity of flavonoids or other antioxidants facilitated the caspase-dependent neuroprotective effect of phytoestrogen, thereby conferring a significant neuroprotective ability against Aβ-mediated toxicity.

Our results demonstrate that inhibition of caspase and scavenging of ROS act cooperatively to save neurons from Aβ-mediated toxicity. Therefore, inhibition of caspase cascade and decrease in the level of ROS are proposed as neuroprotective strategies in AD. Base on these results, the development of neuroprotective agents such as a compound that combines potent antioxidant and caspase inhibitory properties may prevent the incidence of AD or retard the progression of drug discovery for clinical therapies of AD.