Note

Protective Effects of Kaempferol (3,4',5,7-tetrahydroxyflavone) against Amyloid Beta Peptide (Aβ)-Induced Neurotoxicity in ICR Mice

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To determine the effects of kaempferol, rat phaeochromocytoma cells (PC12) and Institute of Cancer Research (ICR) mice were utilized as neuronal models. Using in vitro assays, kaempferol was shown to have protective effects against oxidative stress-induced cytotoxicity in PC12 cells. Administration of kaempferol also significantly reversed amyloid beta peptide (Aβ)-induced impaired performance in a Y-maze test. Taken altogether, the results reported here suggest that further investigation is warranted of the influence of kaempferol on pathways related to Alzheimer’s disease.

Key words: kaempferol (3,4',5,7-tetrahydroxyflavone); Alzheimer’s disease; amyloid beta peptide; oxidative stress

Alzheimer’s disease (AD) is a neurodegenerative disorder of the central nervous system. It is correlated with cognitive malfunction, loss of memory, unusual behavior, and declining perception ability. AD is accompanied by the following pathological changes in the brain: diffuse loss of neurons, abnormal deposits of intracellular proteins, and extracellular proteins such as senile plaques. The most abundant constituent of senile plaques in AD is a 40–42 amino acid peptide named amyloid-beta peptide (Aβ), which is formed by cleavage of the amyloid precursor protein by β- and γ-secretase.1,2) The mechanism and cellular pathway of neuronal degeneration induced by this peptide are not yet fully understood, but Aβ-induced cytotoxicity has been found to be caused by the intracellular accumulation of reactive oxygen species (ROS), eventually resulting in peroxidation of cell membranes, modification of proteins, damage to DNA/RNA, and cell death.3,4) The fact that antioxidants, vitamin E and C, protect neuronal cells in vitro against Aβ-induced cytotoxicity confirms this.3,4) Besides the progressive deposition of Aβ, a deficiency of acetylcholine (Ach), a neurotransmitter found in the synapses of the cerebral cortex, is another prominent feature observed in AD patients. Although the cause of the vulnerability of basal forebrain cholinergic neurons and its relationship to Aβ remain unclear, several studies have reported that acetylcholinesterase promotes the assembly of Aβ into fibrils.5,6)

Flavonoids are polyphenolic compounds, ubiquitous in fruits, that can be classified into six categories: flavones, flavonols, flavanones, isoflavones, flavanols, and anthocyanins.5,6) Epidemiological and pharmacological studies have indicated that consumption of flavonoids is associated with many beneficial effects, including antioxidative, antiviral, anticancer, and anti-inflammatory effects, as well as the prevention of cardiovascular diseases.7–10) Because some of the beneficial effects of flavonoids are believed to result mainly from their antioxidative properties, these antioxidative activities have gained increasing interest.

The objective of this study was to examine the protective activities of kaempferol against Aβ, a major representative flavonol that has been isolated from tea, broccoli, grapefruit, and other plant sources, including medicinal herbs.5,6) To investigate the protective effects of kaempferol against AD, rat phaeochromocytoma (PC12) cells and Aβ-injected ICR (Institute of Cancer Research) mice were utilized as neuronal models.

PC12 cells were cultured and maintained as previously described.11) Although PC12 cell line was originally isolated from adrenal medulla tumors, the adrenal medulla originates in the neural crest, and it displays properties associated with sympatheticblasts as well. Hence, it is widely used in research related to AD.1,2,11) Briefly, RPMI 1640 supplemented with 10% heat inactivated horse serum, 5% fetal bovine serum, and 1% antibiotic-antimycotic was provided as the growth medium in a humidified incubator at 37 °C under 5% CO2. Cultured cells were dislodged from culture dishes, and subcultured when each dish was 80–90% confluent. The split ratio for subculture was 1:4. The medium was changed at least 3 times a week.

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Abbreviations: AAPH, 2,2’-azobis(2-amidinopropane)dihydrochloride; ABTS, 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); Aβ, amyloid beta peptide; Ach, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer’s disease; APP, amyloid precursor protein; DCF-DA, 2’,7’-dichlorodihydrofluorescein diacetate; ICR, Institute of Cancer Research; ICV, intracerebroventricular; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PC12, rat phaeochromocytoma cells; PUF, polyunsaturated fatty acid; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances
Intracellular oxidative stress levels were evaluated by the 2',7'-dichlorofluorescin diacetate (DCF-DA) assay to detect ROS by a method previously described. Briefly, seeded PC12 cells in 96-well plates were pretreated for 48 h at 25 μM, 50 μM, 75 μM, and 100 μM (final concentration) of kaempferol. The cells were treated with and without hydrogen peroxide for 2 h, and then 50 μM of DCF-DA was added. After a 50 min incubation period, DCF was quantified with a fluorometer using a 485 nm excitation filter, and a 535 nm emission filter (GENOis TECAN, Mannedorf, Switzerland).

Blue-green 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals were used to measure the radical scavenging activity of kaempferol. The ABTS radical is of blue-green color when it is in its odd electron state but it loses its color when the electron from kaempferol or vitamin C pairs with its unpaired electron. One mM 2,2′-azobis(2-aminopropane) dihydrochloride, a radical initiator, was added to 2.5 mM ABTS in phosphate buffered saline (PBS; pH 7.4). The mixed solution was heated in a water bath at 68 °C. The ABTS radical solution was adjusted to an absorbance of 0.650 ± 0.020 at 734 nm with additional PBS. Twenty μl of the sample was added to 980 μl of the ABTS solution. The mixture was incubated in a 37 °C water bath without any light. The decrease in absorbance at 734 nm was measured after 10 min of incubation.

Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, as described previously. Briefly, PC12 cells were plated at a density of 104–105 cells/well on 96-well plate and incubated with the concentrations of kaempferol described above for 48 h. After incubation with hydrogen peroxide or Aβ25-35, cell viability was determined by conventional MTT reduction. The amount of MTT formazan crystal that formed in the sample was quantified by measuring the absorbance using a microplate reader (GENOis TECAN, Mannedorf, Switzerland) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

The level of lipid peroxidation was determined by measuring the amount of thiobarbituric acid reactive substances (TBARS) by a modification of the method of Mihara and Uchiyama. Briefly, PC12 cells were pretreated with various concentrations of kaempferol and vitamin C for 48 h. The cells were homogenized with PBS (pH 7.4) after removal of the growth medium, and then mixed with H2PO4 solution (1% v/v) and 160 μl of thiobarbituric acid solution (0.67% w/v). The mixture was heated at 95 °C for 45 min. After cooling, n-butanol was added to the supernatant. The absorbance of the extracted pigment was measured at 532 nm (UV-1601 Shimadzu, Kyoto, Japan). The protein content was determined by the Bradford method using a protein assay kit (Bio-Rad Laboratories, Hercules, CA).

ICR mice (male, 4 weeks old; Daehan Biolink, Chungnam, Korea) were housed nine per cage in a room maintained under a 12 h light-dark cycle, 55% humidity at 23–25 °C. Kaempferol was mixed with a commercial diet at concentrations of 5, 10, 20, and 40 mg/kg of body weight (0.0003, 0.0006, 0.0012, and 0.0024% respectively). The mice had free access to feed and water for 4 weeks, and then Aβ1-42 was administered by intracerebroventricular (ICV) injection to induce memory impairment. The control group was injected with non-toxic reverse Aβ1-42. Aβ was dissolved in 0.85% (v/v) sodium chloride solution and 5 μl (410 pmol/mouse) was injected into each mouse using a Hamilton micro-syringe inserted to a depth of 2.5 mm. All experimental procedures were approved by the guidelines established by the Animal Care and Use Committee of Korea University. Spontaneous alternation behavior in a Y-maze test was recorded to assess immediate working memory performance. The Y-maze test was performed as described previously. Spontaneous alternation of mice was calculated as the ratio of actual alternations to possible alternations (defined as the total number of arm entries minus two), multiplied by 100.

Each result was expressed as the mean ± SD. The statistical significance of differences among groups was calculated by one-way analysis of variance (ANOVA). Data were analyzed by Duncan’s multiple-range test using the Statistical Analysis System (SAS) software package (SAS Institute Inc., Cary, NC). p < 0.05 was considered statistically significant.

Since ROS (e.g., hydrogen peroxide and hydroxyl radical) have been found to mediate cell injury, a DCF-DA assay was performed to evaluate the inhibitory effects of kaempferol against intracellular ROS formation. In this assay, accumulation of ROS in PC12 cells was found to increase by 153.2 ± 2.0% after treatment with 100 μM of hydrogen peroxide over 2 h as compared with the control group (Fig. 1A). Forty-two h of pretreatment of PC12 cells with vitamin C and kaempferol resulted in up to an 83.7 ± 2.8% and a 120.3 ± 1.5% decrease in ROS levels respectively. In addition, the antioxidant activity of kaempferol was examined by measuring ABTS radical scavenging activity; kaempferol quenched radicals in a dose-dependent manner (Fig. 1B; r2 = 0.993), and was more effective than vitamin C. ROS readily damage biological molecules, including lipids, carbohydrates, proteins, and DNA, and this can ultimately lead to apoptotic or necrotic cell death. Increased oxidation of protein and DNA, increased lipid peroxidation, and lowered levels of polyunsaturated fatty acids (PUFAs) have been reported in the AD brain. Aβ has been reported to be a possible source of oxidative stress in the brain of AD patients because it can acquire a free radical state and consequently induce neurotoxicity. Conversely, it was also suggested that oxidative stress activates β-secretase and increases Aβ secretion in late-onset AD. These cleaved Aβs can enter the mitochondria and induce free radicals, which eventually lead to neuronal degeneration in AD. Thus removal of excess ROS or inhibition of their generation by antioxidants might be an effective therapeutic strategy in preventing neurodegenerative disorders. Recently, work has been done to determine the antioxidative effects of kaempferol. In agreement with these studies, hydrogen peroxide-induced deposits of ROS were found to be significantly reduced by the presence of kaempferol in PC12 cells; in contrast, the same concentration of vitamin C was less effective than kaempferol (p < 0.01). In accordance with the results of the DCF-DA assays, kaempferol demonstrated the same pattern of radical quenching activity, which was again higher than that of vitamin C. These results indicate that
kaempferol can exhibit a neuroprotective effect by scavenging radicals directly in PC12 cells, which can be produced from Aβ in AD.

Since yellow tetrazolium salt is metabolically reduced in live cells to form insoluble formazan crystals, the amount of these crystals is directly proportional to the number of viable cells. The cellular reactions in this reduction are not fully understood, but several studies have suggested that the mitochondrial succinate dehydrogenase system of active mitochondria is involved.2,19) The protective effects of kaempferol and vitamin C on the loss of PC12 cell viability induced by hydrogen peroxide or Aβ25-35 were assessed with this assay (Fig. 2A and B). Treatment with 100 µM of hydrogen peroxide and with Aβ25-35 decreased the viability of neuronal cells, by 43.3 ± 2.0% and 40.3 ± 10.2% respectively, as compared with the control group (p < 0.01). For the groups pretreated with kaempferol and vitamin C, cell viability increased in a dose-dependent manner. However, cell viability for the cells pretreated with vitamin C was less than when the same concentrations of kaempferol were used (p < 0.01 for all concentrations treated). These results suggest that kaempferol protects PC12 cells has been possibly by protecting the mitochondria.

Malondialdehyde (MDA), a major product of lipid peroxidation, spectrophotometrically measured by detecting TBARS.20) The level of MDA was higher in cells treated with hydrogen peroxide than with the control (126.8 ± 3.91% and 100.0 ± 0.47% respectively, p < 0.01). Pretreatment of cells with all but the lowest dose of vitamin C and kaempferol resulted in statistically significant reductions in MDA as compared with cells treated only with hydrogen peroxide (Fig. 2C). PUFAs

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**Fig. 1.** Effects of Kaempferol on Oxidative Stress in PC12 Cells and Scavenging of Radicals.

A. The control group was not treated. The hydrogen peroxide group was treated only with 100 µM hydrogen peroxide for 2 h. Sample groups were preincubated with 25 µM, 50 µM, 75 µM, and 100 µM of vitamin C or kaempferol for 48 h before treatment with 100 µM hydrogen peroxide. Levels of oxidative stress in PC12 cells were measured using DCF (2',7'-dichlorofluorescein), as described in “Materials and Methods.” Data represent the mean value (n = 4) ± SD. Duncan’s multiple-range test of SAS indicated a significant difference (*p < 0.01 vs. the control group, **p, ***p < 0.01 vs. the hydrogen peroxide group). B. The relationships between vitamin C and kaempferol and absorbance reduction of the free blue-green ABTS radical at 734 nm. Data represent mean absorbance (n = 4) ± SD.

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**Fig. 2.** Protective Effects of Kaempferol on Cell Viability and Oxidative Stress on PC 12 Cells.

A. The control group was not treated. The hydrogen peroxide group was treated with 100 µM hydrogen peroxide for 2 h. Sample groups were preincubated with 25 µM, 50 µM, 75 µM, and 100 µM of kaempferol before treatment with 100 µM hydrogen peroxide. The same concentrations of vitamin C were also evaluated as a positive control. Data represent the mean (n = 3) ± SD. Duncan’s multiple-range test of SAS indicated a significant difference (*p < 0.01 vs. the control group, **p, ***p < 0.01 vs. the hydrogen peroxide group). B. The control group was not treated. The amyloid beta group was treated with 100 µM Aβ25-35 for 24 h. Sample groups were preincubated with the concentrations described above. The same concentrations of vitamin C were also tested as a positive control. Data represent the mean (n = 4) ± SD. Duncan’s multiple-range test of SAS indicated a significant difference (*p < 0.01 vs. the control group, **p, ***p < 0.01 vs. the amyloid beta group). C. The level of lipid peroxidation was measured after treatment with 100 µM hydrogen peroxide for 2 h. The sample groups and positive control were treated under the conditions described above. Data represent the mean (n = 4) ± SD. Duncan’s multiple-range test of SAS indicated a significant difference (*p < 0.01 vs. the control group, **p, ***p < 0.05 vs. the hydrogen peroxide group).
are abundantly present in brain phospholipids, which are the major constituents of the cellular membrane. They are not only one of the most vulnerable targets for free-radical damage but also the substrate of lipid peroxidation. Lipid peroxidation produces secondary bioactive aldehydes, including 4-hydroxy-2-nonenal and acrolein, which can adduct to DNA and proteins. Increased levels of both aldehydes have been observed in brain regions of AD patients. Moreover, peroxidation of membrane lipids and proteins (carbonyl group formation) can disrupt membrane fluidity, reduce membrane potential, elevate membrane permeability to ions by inactivating membrane-bound enzymes, and eventually lead to cell death. Therefore, the results of this study imply that kaempferol suppresses lipid peroxidation in PC12 cells and thus might be beneficial in protecting cells against oxidative damage.

To confirm the anti-neurotoxic activity of kaempferol, an animal model of Aβ-induced memory deficit, which involves administering an ICV injection of Aβ to ICR mice, was used. Since Aβ1-42 is reported to cause memory impairment in mice, this method of Aβ1-42 exposure is widely used as an in vivo model of Aβ-induced memory deficit. In the mouse model, all the mice treated with the kaempferol mixed diet had normal increases in body weight, and no acute toxicity or liver toxicity was observed (data not shown). Spontaneous alternation behavior, which is used as a measure of spatial working memory, was recorded using the Y-maze test. Administration of Aβ1-42 effectively impaired spatial working memory as compared with the control group (p < 0.01, Table 1). The groups pretreated with kaempferol had an ameliorative Aβ1-42-induced decrease in alternation behavior (50 µM: 66.43 ± 4.03%; 75 µM: 66.32 ± 6.19%; 100 µM: 77.26 ± 6.82%; p < 0.01 for all concentrations). There was no significant difference in the numbers of arm entries for any group. These results indicate that the administration of kaempferol attenuated impaired spatial working memory in the Y-maze test but did not affect the general locomotive activity of the mice. A number of studies have shown that dietary intervention with antioxidants has beneficial effects on cognitive functions. Supplementation with vitamins C and E and plant extracts (strawberry, blueberry, and spinach) improved learning and memory retention in aged rats. In addition, it has also been reported that antioxidant-enriched food reversed age-related changes in antioxidant defenses in neuronal tissues. However, it is still unclear whether the protective effects of dietary flavonoids are associated with their own reducing properties or with other mechanisms, because they are substrates for several enzymes that are located in the small intestine, colon, and liver. Possible modifications of those enzymes include methylation, glucuronidation, and sulfation, and these modifications can alter the antioxidative properties or the lipophilicity of flavonoids, which is positively related to the permeability of flavonoids through the blood-brain barrier. Therefore, it is not clear whether kaempferol diminished the neurotoxicity of Aβ directly or indirectly in the brains of the mice. However, the present data suggest that the administration of kaempferol effectively reversed Aβ-induced damage in the behavior test; thus, it might have contributed to ameliorating or improving the spatial working memory of the mice. To our knowledge, this is the first report that kaempferol has protective activity against Aβ-induced memory impairment in vivo.

The present work clearly indicates that kaempferol exerted protective activity against oxidative stress, which plays a crucial role in the onset of AD. Taken together, these results indicate that kaempferol, which has an antioxidative capacity, might be protective against oxidative-stress-induced neuronal damage, possibly by ameliorating disruption of neuronal cell membranes and mitochondria. Moreover, kaempferol also improved Aβ-induced memory impairment in mice. These actions of kaempferol can account for its therapeutical profile. Based on the results of these in vitro and in vivo studies, further investigation is warranted to determine the effects of kaempferol at the molecular level and to examine its potential for therapeutic use.

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References

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