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Flavonoids Possess Neuroprotective Effects on Cultured Pheochromocytoma PC12 Cells: A Comparison of Different Flavonoids in Activating Estrogenic Effect and in Preventing β-Amyloid-Induced Cell Death

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Despite the classical hormonal effect, estrogen possesses a neuroprotective effect in the brain, which has led many to search for novel treatments for neurodegenerative diseases. Flavonoids, a group of compounds mainly derived from vegetables, share a resemblance, chemically, to estrogen, and indeed, some have been used as estrogen substitutes. To search for potential therapeutic agents against neurodegenerative diseases, different subclasses of flavonoids were analyzed and compared with estrogen. First, the estrogenic activities of these flavonoids were determined by activating the estrogen-responsive elements in cultured MCF-7 breast cancer cells. Second, the neuroprotective effects of flavonoids were revealed by measuring its inhibition effects on the formation of reactive oxygen species, the aggregation of β-amyloid, and the induction of cell death by β-amyloid in cultured neuronal PC12 cells. Among these flavonoids, baicalein, scutellarin, hibifolin, and quercetin-3′-glucoside possessed the strongest effect in neuroprotection; however, the neuroprotective activity did not directly correlate with the estrogenic activity of the flavonoids. Identification of these flavonoids could be very useful in finding potential drugs, or food supplements, for treating Alzheimer's disease.

KEYWORDS: Alzheimer's disease; β-amyloid; flavonoids; estrogen; neuroprotection

INTRODUCTION

Estrogen is one of the key regulators for growth and differentiation, which shows various physiological functions in a wide range of target tissues (*1*). Estrogen exerts its effects via the binding to intracellular estrogen receptors, the phosphorylation of the receptor, the binding of the receptor complex to the promoters of target genes, and subsequently the activation of gene transcription. Estrogen replacement therapy (ERT) is used to treat menopausal symptoms in aged women (*2*); however, the elevation of estrogen level causes adverse effects, e.g., the formation of breast cancer and vaginal bleeding (*3*, *4*).

Clinically, ERT-treated patients experience a reduced risk of suffering from Alzheimer's disease (*5*). This notion is further supported by in vitro experiments that estrogen has neuromodulatory and neuroprotective roles in cultured neurons (*6*−*8*). This raises a new functional role of estrogen despite its classical hormonal effects and helps to develop novel treatments for patients suffering from Alzheimer's disease (*9*). Pathologically, Alzheimer's disease is manifested by selective oxidative stress-induced neuronal cell death, the deposition of β-amyloid (Aβ) peptide into senile plaques in the extracellular space, and the formation of neurofibrillary tangles inside the neuron. Different therapeutic efforts are targeted at blocking Aβ aggregation, Aβ production, Aβ-induced toxicity, and oxidative stress (*10*−*12*). Estrogen has been shown to be effective against oxidative stress and Aβ-induced toxicity in neurons (*6*−*8*); however, the mechanisms underlying the estrogen-mediated neuroprotection have not been fully elucidated.

Flavonoids, a large group of natural compounds, have been considered as substitutes for estrogen (*13*, *14*) and have been proven to possess neuroprotective effects (*15*, *16*). Flavonoids are commonly included in food additives and health food supplements and are also considered as the active ingredients in Chinese herbal medicines. Chemically, flavonoids are divided into different subclasses including flavanones, flavones, flavonols, flavanonols, isoflavones, chalcones, etc. As of today, the estrogenic and neuroprotective effects of different subclasses of flavonoids have never been compared. To search for potential therapeutic agents against neurodegenerative disease, 64 flavonoids, mostly from vegetables and herbal medicines, were screened for (i) the estrogenic activity in human breast cancer MCF-7 cells, (ii) the neuroprotective effect against the formation of reactive oxygen species (ROS) in cultured PC12 cells, and (iii) the prevention of Aβ aggregation and Aβ-induced cytotoxicity in cultured PC12 cells.

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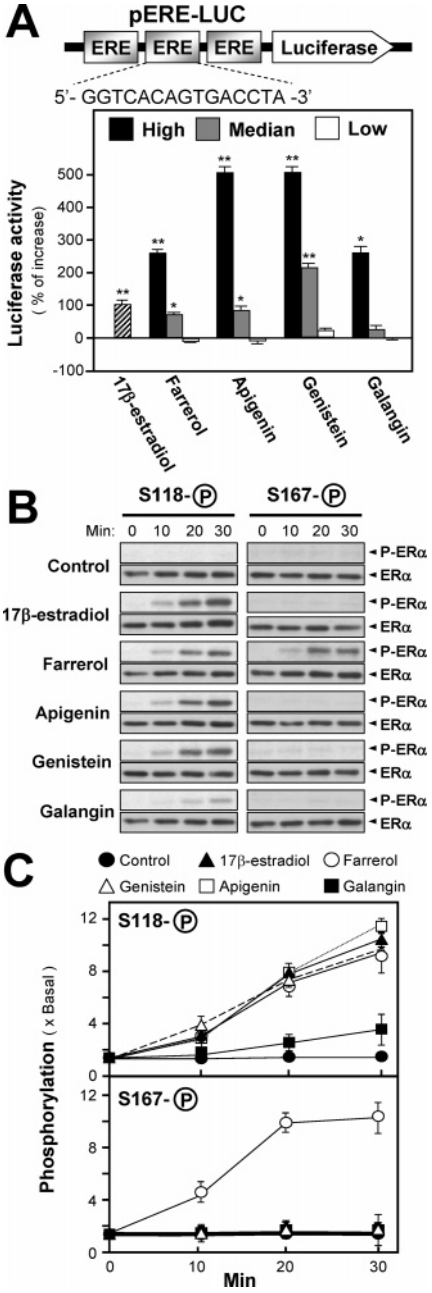

Figure 1. Activation of estrogen receptor by flavonoids in cultured MCF-7 cells. (A) The estrogen responsive reporter construct that contained three repeats of estrogen response element (ERE) was placed upstream of a firefly *luciferase* gene as pERE-Luc (upper panel). In pERE-Luc stably transfected MCF-7 cells, flavonoid was added for 48 h before the assay for luciferase activity (lower panel). The concentrations of flavonoids (farrerol, apigenin, and genistein) were at 10 (high), 1 (median), and 0.1 μM (low); galangin was at 5 (high), 0.5 (median), and 0.05 μM (low). 17β-Estradiol (10 nM) served as a positive control. The activity of pERE-Luc is expressed in percentage of increase relative to the control (without drug). (B) MCF-7 cultures were serum-starved for 3 h before the addition of flavonoids at different times. Farrerol, apigenin, and genistein at 10 μM and galangin at 5 μM were used here. 17β-Estradiol (10 nM) served as a positive control. Total estrogen receptor (ER) α and S118 or S167 phosphorylated ER α (P-ERα; both at $\sim$66 kDa) were revealed by using specific antibodies. (C) The phosphorylations were quantified from the blots in part B by calibrated densitometry. Data are normalized and expressed as the ratio to the basal activity where time 0 (untreated) equals 1. Data are expressed as means $\pm$ SEM, where $n = 4$, each with triplicate samples. *$p < 0.05$ and **$p < 0.01$ as compared to the control group (without drug).

MATERIALS AND METHODS

General Chemicals. 17β-Estradiol, Congo red, vitamin C, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO). Aβ 1−40 was from American Peptide Co. (Sunnyvale, CA); thioflavin was purchased from International Laboratory USA (Las Vegas, NV).

Tested Flavonoids. Naringenine, naringin, phloretin, and (−)-epicatechin were purchased from Sigma. Apigenin, baicalein, baicalin, (−)-catechin, genistein, genistin, kaempferol, puerarin, 4′,7-dimethoxyl-puerarin, 4′,7-diacetyl-puerarin, sulfuretin, tangeretin, and wogonin were purchased from Wakojunyaku (Osaka, Japan). Alpinetin, cardamonin, chrysin, daidzein, daidzin, farrerol, glycitein, glycitin, hesperidin, hyperin, icariin, irisflorentin, isorhamnetin, luteolin, lysionotin, neohesperidin, quercetin, rutin, scutellarin, silybin, tectoridin, and tectorigenin were purchased from National Institute for the Control of Pharmaceutical Biological Products (NICPBP) (Beijing, China). Astragalin, calycosin, calycosin-7-O-glucoside, dihydromyricetin, formononetin, galangin, isorhamnetin-3-O-rutinoside, isovitexin, kaempferol-3-O-rutinoside, kaempferol-3-O-glucoside, ononin, phloridzin, pratensein, prunin, quercetin-3′-O-glucoside, scoparin, and tiliroside were obtained from School of Pharmaceutical Science, Peking University (Beijing, China). Apiin, ginkgetin, hibifolin, isoquercitrin, liquiretin, 8-methoxyl-hebacetin, morusin, and vitexicarpin were obtained from Kunming Institute of Botany, Chinese Academy of Science (Kunming, China). The purities of these compounds were over 98%, and they were dissolved in dimethyl sulfoxide (DMSO) to give stock solutions at concentrations of 25−100 mM. Chemical structures of these flavonoids are given in the Supporting Information.

Cell Culture. Human mammary epithelial carcinoma cell line MCF-7 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown in modified Eagle's medium (MEM), supplemented with 10% fetal bovine serum, 1 mM nonessential amino acids, 0.1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified CO_2 (5%) incubator at 37 °C. Reagents for cell cultures were purchased from Invitrogen Technologies (Carlsbad, CA). Before plating, phosphate-buffered saline (PBS) was used to wash the cells, and the medium was changed to MEM-α (phenol red-free) containing 5% charcoal dextran-treated fetal bovine serum for 2 days. MCF-7 cells were seeded in a 24 well plate at 25000 cells/cm^2. The cells were treated with different samples for the appropriate times and concentrations. The rat pheochromatocytoma PC12 cell was obtained from ATCC and maintained in Dulbecco's modified Eagle's medium supplemented with 6% fetal bovine serum and 6% horse serum at 37 °C in a water-saturated 7.5% CO_2 incubator. All reagents for cell cultures were purchased from Invitrogen.

Estrogen Promoter Assay in MCF-7 Cells. Three repeats of estrogen-responsive elements (5′-GGT CAC AGT GAC C-3′) were synthesized as described previously (*17*) and then subcloned into pTAL-Luc, a promoter−reporter vector (Clontech, Mountain View, CA) that has a down stream reporter of firefly *luciferase* gene; this DNA construct was named pERE-Luc. A stable cell line of MCF-7 was established by cotransfection of pERE-Luc and pcDNA3 under selection of 500 μg/mL G418. To determine the estrogenic activity, stable pERE-Luc-expressed MCF-7 cells in a 24 well plate were treated with 10 μM estrogen or different doses of flavonoids for 48 h. Cultures were then collected by lysis buffer containing 0.2% Triton X-100, 1 mM dithiothreitol, and 100 mM potassium phosphate buffer (pH 7.8) and subjected to luciferase and protein assays. The luciferase assay was performed by a commercial kit (Tropix Inc., Bedford, MA). The readings corresponding to luciferase were quantified by FLUOstar Optima (BMG Labtech, Offenburg, Germany), where luciferase activity was normalized per mg of protein in each sample.

Determination of Estrogen Receptor Phosphorylation. The phosphorylations of estrogen receptor at serine 118 (S118) and serine 167 (S167) were determined by Western blotting. Cultures were serum-starved for 3 h before the drug applications. After drug treatments, cultures were collected immediately in lysis buffer [125 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 200 mM 2-mercaptoethanol, pH 6.8] and SDS−polyacrylamide gel electro-

Table 1. Estrogenic and Neuroprotective Activities of the Selected Flavonoids[a]

I: Estrogenic activity	II: Anti-oxidation	III: Anti-Aβ aggregation	IV: Anti-Aβ-induced cytotoxicity
+ > 10%	+ > 10%	+ > 10%	+ > 10%
++ > 100%	++ > 20%	++ > 30%	++ > 30%
+++ > 300%	+++ > 30%	+++ > 50%	
++++ > 500%		++++ > 70%	

biochemical tests	I	II	III	IV	biochemical tests	I	II	III	IV
				flavanones					
alpinetin	+	–	–	–	naringenin	+	–	–	–
farrerol	++	+	++	–	naringin	–	–	–	–
hesperidin	–	–	–	–	neohesperidin	–	+	–	–
liquiretin	+	+	–	–	prunin	+	–	–	–
				flavones					
apigenin[b]	++++	++	–	–	luteolin[b]	++	–	+	–
apiin	–	–	–	–	lysionotin[b]	+++	++	–	+
baicalein[b]	++	–	+++	++	morusin[b]	–	–	+	–
baicalin	+	–	+	+	scoparin	–	–	–	–
chrysin[c]	+	–	–	–	scutellarin	+	–	++	++
8-methoxyl-hebacetin	+	+	+	–	tangeretin	+	–	–	–
isovitexin	–	+	–	–	wogonin[b]	–	–	–	–
				chalcones					
cardamonin[b]	–	+	–	–					
				dihydrochalcones					
phloretin[b]	+	+	–	–	phloridzin[b]	–	+	–	–
				flavanonols					
dihydromyricetin[b]	–	–	+	+	silybin	+	++	+	–
				flavanes					
(–)-catechin	–	–	+	+	(–)-epicatechin	–	–	+	+
				aurones					
sulfuretin[b]	+	+	+	+					
				biflavones					
ginkgetin[b]	–	+	+	–					
				isoflavones					
calycosin[b]	+++	–	–	–	irisflorentin	+++	+	–	–
calycosin–7-O-glucoside	++	–	–	–	ononin	++	+	+	+
daidzein	++	+	++	+	pratensein	+++	+	–	–
daidzin	++	–	+	–	puerarin[b]	+	+	–	–
formononetin[c]	++	++	–	–	4′,7-dimethoxyl-puerarin	–	+	–	–
genistein[b]	++++	–	–	–	4′,7-diacetyl-puerarin	–	+	–	–
genistin	+++	–	–	–	tectoridin	++	–	–	–
glycitein[b]	++	–	–	–	tectorigenin	+++	–	–	–
glycitin	++	+	++	+					
				flavonols					
astragalin	–	–	–	–	kaempferol	++	+	++	+
galangin[b]	++	–	–	–	kaempferol-3-O-glucoside	+	–	+	+
hibifolin	–	+	+++	++	kaempferol-3-O-rutinoside	–	+	+	–
hyperin	–	+	+	–	quercetin[b]	+	++	+	–
icariin[b]	–	–	–	–	quercetin–3′-O-glucoside	+	++	+++	+
isoquercitrin	–	–	+	+	rutin	–	–	+	–
isorhamnetin	–	+	–	–	tiliroside[b]	–	–	++	–
isorhamnetin--3-O-rutinoside	–	–	–	+	vitexicarpin[b]	–	–	–	–
17β-**estradiol**	+	+	+	+					
vitamin C	ND	+++	ND	ND					
Congo red	ND	ND	++++	++					

[a] Data are means ± SEM, were $n = 3$–6, each with triplicate samples. The value of SEM is within 5% of the mean, which is not shown for clarity. + to ++++ indicate the ranking of the estrogenic activity (I), antioxidation (II), anti-Aβ-aggregation (III), and anti-Aβ-induced cytotoxicity (IV). – indicates no effect, i.e., below 10% increase in the tested activities. ND indicates not determined. The working concentrations of 17β-estradiol, vitamin C, and congo red were 10 nM, 500 μM, and 1 μM, respectively. The submaximum dose of these drugs is used for comparison. [b] In general, three concentrations (50, 5, and 0.5 μM) were used except 10, 1, and 0.1 μM. The ranking is based on the maximum dose. [c] In general, three concentrations (50, 5, and 0.5 μM) were used except 1000, 100, and 10 nM. The ranking is based on the maximum dose.

phoresis was performed. After they were transferred, the membrane was incubated with anti-phospho-estrogen receptor α S118 (Upstate, Lake Placid, NY) at 1:2000 dilution or anti-phospho-estrogen receptor α S167 at 1:1000 dilution (Upstate) at 4 °C for 12 h. Followed by incubation of horseradish peroxidase-conjugated anti-rabbit secondary antibodies in 1:5000 dilution for 1 h at room temperature, the immunocomplexes were visualized by the enhanced chemiluminescence (ECL) method (Amersham Biosciences, Piscataway, NJ). The band intensities, recognized by the antibodies in the ECL film, in control and agonist-stimulated samples were run on the same gel and under strictly standardized ECL conditions including the amounts of primary and secondary antibodies, the incubation time for antibodies, the amount of ECL chemicals, and the time for image development. The bands were compared on an image analyzer, using in each case a calibration

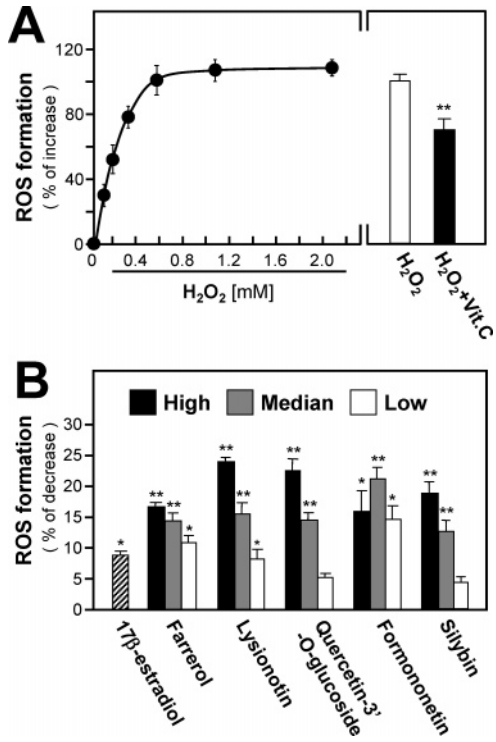

Figure 2. Flavonoids inhibit H_2O_2-induced ROS formation in PC12 cells. (A) Cultured PC12 cells were exposed to H_2O_2 (0–2 mM) for 1 h. The level of intracellular ROS was measured by DCF. The pretreatment of vitamin C (0.5 mM) for 24 h inhibited H_2O_2 (0.4 mM)-induced ROS formation, which served as a positive control. The results are in percentage of increase in ROS formation relative to the control (without H_2O_2). (B) Cultured PC12 cells were pretreated with flavonoids for 24 h and then exposed to H_2O_2 (0.4 mM) for 1 h. Farrerol, quercetin-3′-O-glucoside, and silybin at 50 (high), 5 (median), and 0.5 μM (low) were used. Lysionotin and formononetin at 10 (high), 1 (median), and 0.1 μM (low) were used. 17β-Estradiol (10 nM) was used for comparison. The results are in percentage of inhibitory effect against ROS formation relative to the control (with H_2O_2 alone). Data are expressed as means ± SEM, where $n = 4$, each with triplicate samples. *$p < 0.05$ and **$p < 0.01$ as compared to the control group (with H_2O_2 alone).

plot constructed from a parallel gel with serial dilution of one of those samples; this was to ensure the subsaturation of the gel exposure.

Determination of ROS Formation. The determination of ROS level in cell cultures was performed according to ref *18*, with minor modifications. Cultured PC12 cells in a 96 well plate were pretreated with different flavonoids for 24 h and labeled by 100 μM DCFH-DA (Sigma) in HBSS for 1 h at room temperature. Cultures were then treated with 0.4 mM hydrogen peroxide (H_2O_2) for 1 h. The amount of intracellular H_2O_2-induced ROS was detected by fluorometric measurement with excitation at 485 nm and emission at 530 nm (Spectra max Gemini XS, Molecular Devices Corp., Sunnyvale, CA)

Determination of Aβ Aggregation and Its Cytotoxicity. Aβ (1−40) peptide at a final concentration of 10 μM together with different flavonoids were dissolved in water and incubated for 4 days at 37 °C according to previous report (*19*). A 50 μL sample was mixed with 150 μL of thioflavin solution (5 μM in 50 mM glycine, pH 8.5) to determine Aβ aggregation by measuring the fluorescence intensity at emission (435 nm) and excitation (480 nm) (Spectra max Gemini XS) (*20*). In the Aβ-induced cytotoxicity test, cultured PC12 cells in a 96 well plate were treated with aged Aβ (4 days at 37 °C) for 24 h. Followed by addition of MTT (Sigma) in PBS at a final concentration of 0.5 mg/mL for 2 h, the medium was aspirated, and the cultures were resuspended by 150 μL of DMSO to determine the cell viability by measuring the absorbance at 570 nm.

Other Assays. Protein concentrations were measured routinely by Bradford's method with a kit from Bio-Rad Laboratories (Hercules, CA). Statistical tests were done by using one-way analysis of variance. The data were separated into two groups to compare the activation of both drugs over the control. The control group was varied in different experiments, which was specified in the figure legends. Data were expressed as means ± standard errors of the mean (SEM), where $n = 4$. Statistically significant changes were classified as significant (*) where $p < 0.05$ and highly significant (**) where $p < 0.01$.

RESULTS

Estrogenic Activities. To investigate the estrogenic activity, a human breast cancer MCF-7 cell line stably transfected with 3× ERE-Luc reporter (pERE-Luc; **Figure 1A**, upper panel) was employed. MCF-7 is a well-known cell line in testing the estrogen response. The luciferase promoter−reporter (pERE-Luc) system was used here to quantify the estrogenic activity via the activation of estrogen-responsive elements. Sixty-four flavonoids were screened by this assay system. These flavonoids are mainly derived from health foods and Chinese herbal medicines. In addition, these flavonoids have been considered to be active ingredients in foods or herbs. Quantification data of the estrogenic activity of the screened flavonoids were summarized in **Table 1**. Over half of the tested flavonoids possessed activation on pERE-Luc activity. Isoflavone, one of the subclasses of flavonoids and considered a phytoestrogen, showed the strongest activation; these results are in accord with previous reports (*14*). Within this subclass, calycosin, daidzein, formononetin, genistein, and pratensein possessed the strongest activities.

Under each of the subclasses, the one providing the best activation was chosen for further analysis, which included farrerol (flavanone), apigenin (flavone), genistein (isoflavone), and galangin (flavonol). Application of the flavonoids in pERE-Luc-expressing MCF-7 cells induced the transcriptional activity of pERE-Luc in a dose-dependent manner (**Figure 1A**). Apigenin and genistein induced pERE-Luc activity by over three-fold as compared to that of 17β-estradiol (a positive control).

The activation of estrogen-mediated transcription requires the phosphorylation of estrogen receptor, either α- or β-forms (*2*). To validate the estrogenic activity of those screened flavonoids, the phosphorylation of estrogen receptor α was determined by using antibodies that reacted with phosphoestrogen receptor α at the S118 position and total estrogen receptor α. Cultured MCF-7 cells were treated with different flavonoids at different times, and then, the cells were collected to perform Western blot analysis. Results revealed that those chosen flavonoids induced the phosphorylation of estrogen receptor α at the S118 position (~66 kDa) in a time-dependent manner (**Figure 1B**,**C**); the activation was maintained for 30 min after the flavonoid challenge. The flavonoid-induced phosphorylation of estrogen receptor could be increased over 10-fold, except for galangin. The degree of S118 phosphorylation, induced by flavonoids, was in parallel to its activities in inducing pERE-Luc. 17β-Estradiol served as a positive control. In all cases, the total amount of estrogen receptor α (~66 kDa) was unchanged.

Apart from S118 phosphorylation, we also chose to investigate the S167 phosphorylation in estrogen receptor α. Although the requirement of this S167 phosphorylation for estrogen activation is controversial today, the specific phosphorylation still provides another line of evidence to support the function of the flavonoids on this receptor. In cultured MCF-7 cells, the phosphorylation of estrogen receptor α at S167 was insensitive to different flavonoids and 17β-estradiol. In contrast, farrerol showed a specific activation at S167 phosphorylation; the

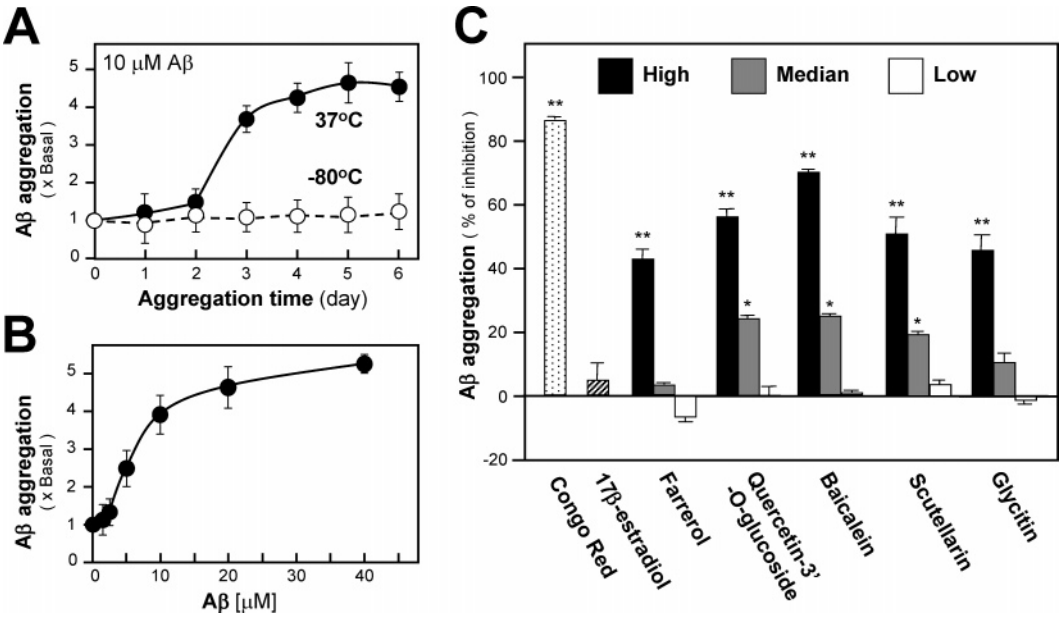

Figure 3. Flavonoids inhibit the aggregation of Aβ. The aggregation of Aβ in an in vitro system was measured by the thioflavin-binding assay. The fluorescence intensity at emission (435 nm) and excitation (480 nm) was measured as described in the Materials and Methods. (A) Aβ (10 μM) was incubated at 37 and −80 °C for 0–6 days. (B) Aβ (0–40 μM) was incubated at 37 °C for 4 days. (C) Aβ (10 μM) was incubated with the flavonoids at 37 °C for 4 days, and then, the inhibition on Aβ aggregation was measured. Farrerol, quercetin-3′-O-glucoside, scutellarin, and glycitin at 50 (high), 5 (median), and 0.5 μM (low) were used, while baicalein at 10 (high), 1 (median), and 0.1 μM (low) were used. 17β-Estradiol (10 nM) and congo red (1 μM) were used for comparison. The results in parts A and B are expressed as the ratio to the basal activity where the control (without Aβ) equals 1. The results in part C are the percentage of inhibition against Aβ aggregation relative to the control (with Aβ but no drug). Data are expressed as means ± SEM, where $n = 4$, each with triplicate samples. *$p < 0.05$ and **$p < 0.01$ as compared to the control group (with Aβ but no drug).

induction was in a time-dependent manner, and the maximum, over 10-fold, was revealed at 20 min after the challenge of farrerol (**Figure 1B**,**C**). The responsive times of farrerol-induced S118 and S167 phosphorylations were similar.

Inhibition of H_2O_2-Induced ROS Formation. The formation of ROS is one of the crucial causes in inducing neuronal cell death. By determining the formation of ROS in H_2O_2-treated cultured PC12 cells, the neuroprotective effects of different subclasses of flavonoids were analyzed. The cultured PC12 cell is a well-established model in studying the neuronal toxicity (*21*). Application of H_2O_2 in cultured PC12 cells induced the formation of ROS in a dose-dependent manner, and H_2O_2-induced ROS formation was markedly reduced by the pretreatment of vitamin C (a known antioxidant) (**Figure 2A**).

In PC12 cultures, flavonoids were applied before the addition of H_2O_2, and then, the cultures were subjected to the determination of intracellular ROS formation. **Table 1** shows the summary of the results. Although many flavonoids showed a reduction of H_2O_2-induced ROS formation, the potency was much lower than that of vitamin C. Selected subclasses of flavonoids were further analyzed. When compared to the control, several flavonoids such as farrerol, lysionotin, quercetin-3′-glucoside, formononetin, and silybin showed the inhibitory effect of H_2O_2-induced ROS formation in a dose-dependent manner (**Figure 2B**). 17β-Estradiol showed weak antioxidation activity in this assay.

Inhibition of Aβ Aggregation and Aβ-Induced Toxicity. The effects of flavonoids in preventing Aβ aggregation in vitro and Aβ-induced cytotoxicity in cultured PC12 cells were determined. Aβ is a crucial factor in inducing neuronal cell death in Alzheimer's disease. The formation of Aβ aggregate after the aging process is believed to be required in Aβ-induced neuronal toxicity. At 37 °C, Aβ aggregate was formed after 2 days of incubation, and the number of aggregate was peaked at day 4 of aging (**Figure 3A**). Under low temperature (−80 °C), the aggregation of Aβ was insignificant. In addition, the aging process of Aβ showed a dose-dependent manner (**Figure 3B**).

In the aggregation assay, Congo red inhibited Aβ aggregation by 85% (**Figure 3C**); this was in accord with a previous study and served as a positive control (*19*). The treatment of 17β-estradiol did not significantly affect the aggregation. **Table 1** shows that the flavonoids possess the inhibitory effect on Aβ aggregation. The subclass of flavonols showed the strongest inhibitory effect including hibifolin, kaempferol, quercetin-3′-O-glucoside, and tiliroside. Representative members of different subclasses were chosen for further analyses. **Figure 3C** shows the inhibitory effects of farrerol, quercetin-3′-O-glucoside, baicalein, scutellarin, and glycitin. The flavonoid-inhibited Aβ aggregation showed a dose-dependent manner, and baicalein, among all the tested flavonoids, contained the strongest inhibitory effect (**Figure 3C**).

Aβ aggregate is toxic to cultured neurons. Application of aged Aβ induced the death of PC12 cells in a dose- and time-dependent manner (**Figure 4A**,**B**). The application of unaggregated Aβ (e.g., at −80 °C) did not show any significant increase of cell death. Many flavonoids prevented the aggregation of Aβ (**Table 1**). For a positive control, the treatment of Congo red inhibited Aβ-induced toxicity by 36%, and the treatment of 17β-estradiol reduced the Aβ-induced cell death by about 20% (**Figure 4C**). The selected flavonoids such as baicalein, scutellarin, glycitin, and hibifolin exerted different protective effects on the Aβ-induced cytotoxicity; the effect was in a dose-dependent manner (**Figure 4C**). The protective effects of these flavonoids, in most cases, were better than that of Congo red. Here, there are two folds of effects in the cotreatment analysis, i.e., Aβ aggregation and Aβ-induced cell death. To eliminate the effect of flavonoid in Aβ aggregation, PC12 cultures were pretreated with different flavonoids before the addition of toxic-aged Aβ. In accord to the results in the cotreatment studies, the pretreatment of flavonoid reduced the Aβ-induced cell death,

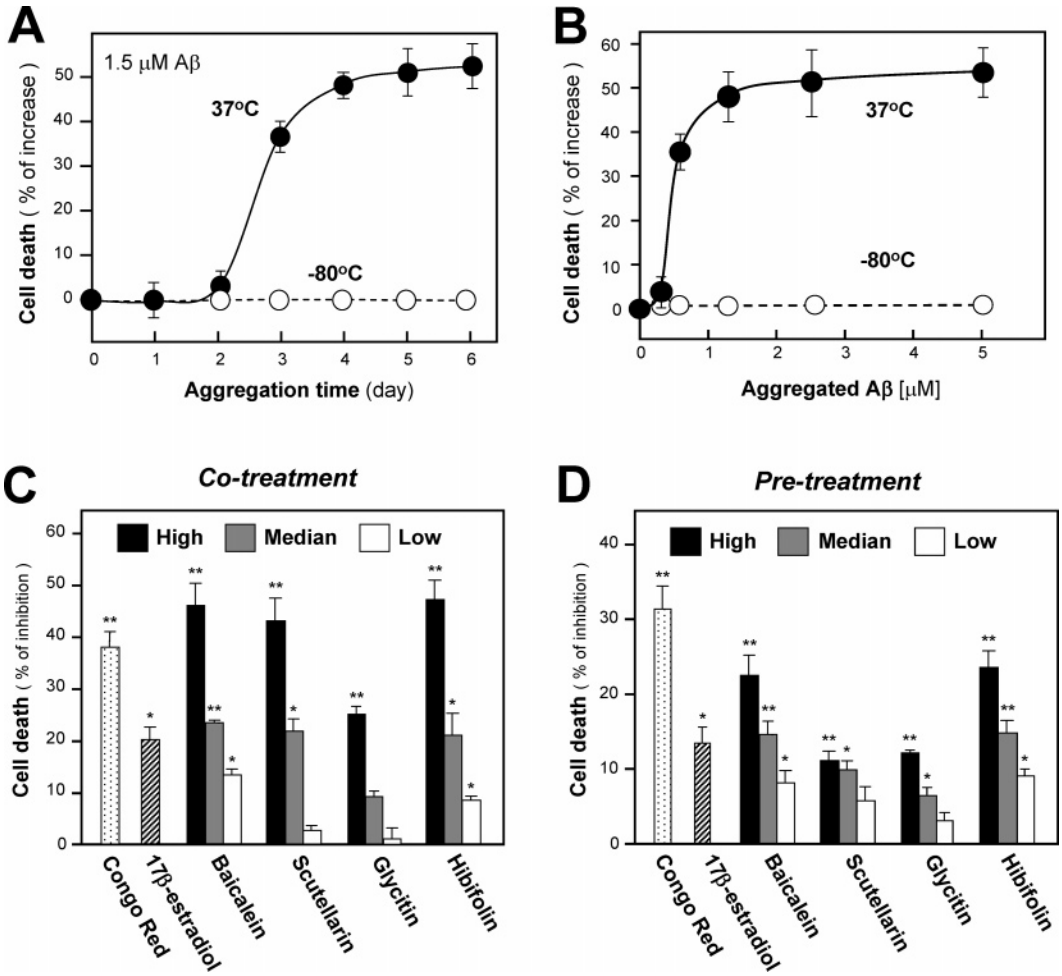

Figure 4. Flavonoids prevent Aβ-induced cytotoxicity in PC12 cells. (A) Aβ (1.5 μM) was aged at 37 and −80 °C for 0–6 days before the application to cultured PC12 cells. The treatment of aged Aβ in cell cultures was 24 h, and the cell viability was measured by MTT assay. (B) Aβ (0–40 μM) was incubated at 37 °C for 4 days before the application to the cultured PC12 cells. The treatment of aged Aβ in cell cultures was 24 h, and the cell viability was measured by MTT assay. (C) Aβ (1.5 μM) was incubated with the flavonoids at 37 °C for 4 days. Cultured PC12 cells were treated with a mixture of aged Aβ and flavonoids for 24 h before the cell viability assay. Scutellarin, glycitin, and hibifolin at 50 (high), 5 (median), and 0.5 μM (low) were used, while baicalein at 10 (high), 1 (median), and 0.1 μM (low) was used. 17β-Estradiol (10 nM) and congo red (1 μM) were used for comparison. (D) Cultured PC12 cells were pretreated with the flavonoids, at the same concentration as in part C, for 3 h, which were washed away. Aβ (1.5 μM), aged at 37 °C for 4 days, was applied onto cultured PC 12 cells for 24 h before the cell viability assay. The results in parts A and B are percentages of increase relative to the control (without Aβ). The results in parts C and D are percentages of inhibition against Aβ-induced cell death relative to the control (with Aβ but no drug). Data are expressed as means ± SEM, where $n = 4$, each with triplicate samples. *$p < 0.05$ and **$p < 0.01$ as compared to the control group (with Aβ but no drug).

except that the degree of reduction was lower (**Figure 4D**). The higher protection effect revealed in the cotreatment of flavonoid could be explained by the dual activities in preventing Aβ aggregation and Aβ-induced cell death.

DISCUSSION

The neuroprotective effect of estrogen has been widely documented in Alzheimer's and Parkinson's diseases. Although estrogen is known to exert direct effects on the brain, the molecular mechanisms implicated in the protective actions on the brain are not fully understood (*2*, *22*). Because of the side effects of estrogen in clinical evidence, the naturally occurring flavonoids are expected to be substitutes for estrogen. In particular, phytoestrogen has been commonly consumed in humans as a health food supplement or as a food additive. Unlike estrogen, flavonoids cannot induce cancer (*3*, *14*). More important, a high concentration of phytoestrogen significantly inhibits the proliferation of cancer cell growth (*23*). In view of the potential usage of flavonoids as a health food supplement, we compared the biological activities of major subclasses of flavonoids in (i) the activation of estrogen promoter and the phosphorylation of estrogen receptor, (ii) the prevention of ROS formation, (iii) the inhibition of Aβ aggregation, and (iv) the protection against Aβ-induced neuronal cell death. The results indicated that the estrogenic properties of flavonoids did not match with its activities in antioxidation, anti-Aβ aggregation, and anti-Aβ-induced cell death.

In the current study, the neuroprotective effects of flavonoids could be revealed in three levels: antioxidation, anti-Aβ aggregation, and anti-Aβ-induced cell death. However, the three activities did not have a direct corelationship; that is, some flavonoids have strong activity in antioxidation but show no effect in Aβ aggregation and/or anti-Aβ-induced cell death. For instance, our results showed that apigenin, lysionotin, silybin, formononetin, quercetin, and quercetin-3′-glucoside had a strong activity in reducing ROS formation in H_2O_2-treated PC12 cells. In contrast, these flavonoids showed weak, or even undetectable, activity in the prevention of Aβ-induced cell death. In opposite,

baicalein, scutellarin, hibifolin, and quercetin-3′-glucoside had a strong activity in the prevention of Aβ-induced cell death but not as an antioxidant. Thus, the antioxidative property of flavonoids is distinct to that of its effect in the prevention of cell death. Moreover, there is only the subclass of isoflavones where most of the members have estrogenic properties. In contrast, the activities in different subclasses are rather diverse. Thus, it is hard to have a conclusion on the structure−function relationship of these flavonoids.

Several studies suggest that estrogen receptors could be involved in estrogen- or flavonoid-mediated neuroprotection (*2*, *24*, *25*). Opposite to this notion, most of the isoflavones having the strongest estrogenic activities, as shown here, did not exert a good neuroprotective effect. The neuroprotective effect of the flavonoids could not be fully explained by the estrogenic effect, which is not the only factor involved in neuroprotection (*26*, *27*). The inhibition of ROS formation is another possible mechanism to explain the neuroprotective effect of flavonoid. As shown in **Table 1**, the strong antioxidants could have weak neuroprotective effects. Thus, the scavenging of ROS by flavonoid could be just one of the factors in contributing to the neuroprotection. The activation of mitogen-activated protein (MAP) kinases, mediated by estrogen, is another possible explanation for the neuroprotective effect. Estrogen is known to activate different MAP kinases; the activation leads to an increase of Bcl-2 (an antiapoptotic gene) expression (*28*, *29*). In accord to this antiapoptotic effect of estrogen, the blockage of these kinases results in the reduction of the insult-induced cell death. However, the role of flavonoids in MAP kinase activation has not been fully revealed. In line with this hypothesis, our recent results showed that the application of flavonoids in cultured neurons could activate the phosphorylation of MAP kinases (Zhu et al. Unpublished results). Whether this activation could lead to the up-regulation of antiapoptotic genes, however, has not been demonstrated.

By means of the luciferase promoter−reporter system, we found that many flavonoids were able to stimulate the transcriptional activity of pERE-Luc and to induce the S118 phosphorylation on estrogen receptor α. These two properties seem to be parallel to each other. In contrast, the S167 phosphorylation is different from the classical signaling pathway mediated by estrogen, which, indeed, is insensitive to 17β-estradiol. This S167 of estrogen receptor α has been shown to be a target for MAP kinases (*30*). From our results, farrerol is the only tested flavonoid that phosphorylates both sites on S118 and S167 of the estrogen receptor. However, this S167 phosphorylation might not be related to the neuroprotection since farrerol did not show prevention against Aβ-induced cell death.

Baicalein and scutellarin are the two strong effectors in preventing Aβ-induced neuronal death. The two chemicals are the major ingredients of a commonly used Chinese medicine—Radix Scutellariae (roots of *Scutellaria baicalensis*). Radix Scutellariae is being used as an anti-inflammatory agent. In line with its function, anti-inflammatory agents have been proposed to treat neurodegenerative diseases (*31*). On the other hand, baicalein was shown to exert neuroprotective effects against glutamate/NMDA stimulation, glucose deprivation, oxidative stress, Aβ-induced toxicity, and inflammation-mediated degeneration (*32*, *33*). Breviscapine, a commercial available extract from roots of *Epigeron breviscapus*, is used to improve learning and memory and to protect brain injury (*34*), while scutellarin is the major compound within Breviscapine. In addition, scutellatin could inhibit NO production in early stages of neuron damage (*18*).

The flavonoids are polyphenolic compounds with multiple biological functions, and they are widely existent in herbal medicines, in fruits, and in vegetables. Our report provides motivation for a more detailed analysis at the individual flavonoid uptake and metabolism in humans and thus to better assess their potential benefits and risks. Although these flavonoids have not been tested in animals, the identification of these neuroprotective agents in cell cultures could be very useful in finding potential drugs for treating Alzheimer's disease. In addition, the synergetic or the combined effects of these flavonoids could also be considered in having a drug that has a broad range of bioactivity.

Supporting Information Available: Chemical structures of flavonoids. This material is available free of charge via the Internet at http://pubs.acs.org.

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