Luteolin Shows an Antidepressant-Like Effect via Suppressing Endoplasmic Reticulum Stress

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Depression is a significant public health problem and some reports indicate an association between depression and endoplasmic reticulum stress. Luteolin is a flavonoid contained in many plants and with a variety of pharmacological properties such as anti-inflammatory, anti-anxiety, and memory-improving effects, suggesting that luteolin penetrates into the brain. In the present study, we investigated the effects of luteolin on endoplasmic reticulum stress-induced neuronal cell death. Luteolin significantly suppressed tunicamycin-induced cell death at 1 to 10 μM in human neuroblastoma cells. Luteolin increased in the expression of the 78 kDa glucose-regulated protein and 94 kDa glucose-regulated protein and decreased in the cleavage activation of caspase-3. Additionally, to investigate whether chronic luteolin treatment has an antidepressive effect, we performed some behavioral tests. Chronic luteolin treatment showed antidepressant-like effects in behavioral tests and, luteolin attenuated the expression of endoplasmic stress-related proteins in the hippocampus of corticosterone-treated depression model mice. These findings indicate that luteolin has antidepressant-like effects, partly due to the suppression of endoplasmic reticulum stress.

Key words depression; endoplasmic reticulum stress; luteolin

In terms of mental illnesses, depression has an enormous influence in society. Major symptoms of depression are a “depressed mood” and “loss of interest and joy.” Despite recent advances in the knowledge related to brain development and function, the mechanisms underlying the pathogenesis of depression remain unclear.

The endoplasmic reticulum is a principal site for protein synthesis, folding, and calcium signaling.1 It is highly sensitive to alterations in calcium homeostasis and perturbations in its environment. A condition that impaired the function of the endoplasmic reticulum, collectively designated “endoplasmic reticulum stress,” can lead to an accumulation of unfolded proteins in the endoplasmic reticulum lumen.2 In response to endoplasmic reticulum stress, cells have developed a self-protective signal pathway termed the unfolded protein response (UPR), leading to induction of molecular chaperones such as 78 kDa glucose-regulated protein (GRP78) and 94 kDa glucose-regulated protein and decreased in the cleavage activation of caspase-3. Additionally, to investigate whether chronic luteolin treatment has an antidepressive effect, we performed some behavioral tests. Chronic luteolin treatment showed antidepressant-like effects in behavioral tests and, luteolin attenuated the expression of endoplasmic stress-related proteins in the hippocampus of corticosterone-treated depression model mice. These findings indicate that luteolin has antidepressant-like effects, partly due to the suppression of endoplasmic reticulum stress.

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In this study, we investigated the antidepressive effects of luteolin from the green pepper leaf, as well as its mechanism, using cell death assay induced by endoplasmic reticulum stress in human neuroblastoma (SH-SY5Y) cells and animal models of depression.

MATERIALS AND METHODS

Purification of Luteolin Luteolin aglycon (luteolin) was provided by Theravalues (Tokyo, Japan). The purity of luteolin was 80%. In the experiments, it was dissolved in phosphate-buffered saline containing 1% dimethyl sulfoxide (DMSO) or suspended in the 0.5% carboxymethylcellulose (Wako, Osaka, Japan).

Cell Culture SH-SY5Y cells were purchased from the European Collection of Cell Culture (Wiltshire, U.K.) and maintained in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, St. Louis, MO, U.S.A.) containing 10% fetal bovine serum (VALEANT, Costa Mesa, CA, U.S.A.), 100 units/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere containing 5% CO2 at 37°C. Cells were passaged by trypsinization every 2—3 d.

Cell Death Assay SH-SY5Y cells were seeded at 1×104 cells per well into a 96-well plate and then incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO2. The entire medium was then replaced with fresh medium containing 1% fetal bovine serum, and each compound (phosphate-buffered saline contained 1% DMSO as a vehicle or luteolin) was added 10 μl/well and pretreated for 1 h, followed by the addition of 2.0 μg/ml tunicamycin. Nuclear staining assay was carried out after a further 27 h of incubation. Cell death...
was assessed on the basis of combination staining with Hoechst 33342 (Molecular Probes, Eugene, OR, U.S.A.) and propidium iodide (Molecular Probes). At the end of the culture period, Hoechst 33342 and propidium iodide dyes were added to the culture medium (at 8, 0.2 μM, respectively) for 15 min. Images were collected using an Olympus IX70 inverted epifluorescence microscope (Olympus, Tokyo, Japan). A total of at least 300 cells per condition were counted in a blind manner by a single observer (M.I.).

**Western Blot Analysis** SH-SY5Y cells were lysed using a cell-lysis buffer (RIPA buffer; Sigma-Aldrich) with a protease inhibitor (Sigma-Aldrich), phosphatase inhibitor cocktails (Sigma-Aldrich), and 1 mM ethylenediaminetetraacetate (EDTA). Mice were decapitated under deep anesthesia induced by sodium pentobarbital (80 mg/kg, intraperitoneally administered (i.p.); Nacalai Tesque, Kyoto, Japan) and their brains quickly removed from the skull, briefly washed in ice-cold saline, and laid on a cooled (4°C) metal plate, on which the brains were rapidly dissected to separate the hippocampus. Brain samples were homogenized in 10 ml/g of tissue of ice-cold lysis buffer (RIPA buffer; Sigma-Aldrich) with protease inhibitor (Sigma-Aldrich), phosphatase inhibitor cocktails (Sigma-Aldrich), and 1 mM of EDTA using a homogenizer (Phycocyan; Microtec Co., Ltd., Chiba, Japan). Lysates were centrifuged at 12000×g for 15 min at 4°C. Supernatants were collected and boiled for 5 min in sodium dodecyl sulfate (SDS) sample buffer (Wako). Equal amounts of protein were subjected to 5 to 20% SDS-polyacrylamide gel electrophoresis (PAGE) gradient gel and then transferred to poly (vinylidene difluoride) membranes (Immobilon-P; Millipore, Bedford, MA, U.S.A.). After blocking with Block Ace (Snow Brand Milk Products Co., Ltd., Tokyo, Japan) for 30 min, the membranes were incubated with the primary antibody. The primary antibodies used were as follows: mouse anti-BiP antibody (BD Bioscience, San Jose, CA, U.S.A.), mouse anti-KDEL antibody (Stressgen Bioreagents Limited Partnership, Victoria, B.C., Canada), rabbit anti-cleaved caspase-3 antibody (Cell Signaling, Beverly, MA, U.S.A.), mouse anti-CHOP antibody (Santa Cruz, Santa Cruz, CA, U.S.A.) and rabbit anti-actin antibody (Sigma-Aldrich). Subsequently, the membrane was incubated with the secondary antibody (goat anti-rabbit or goat antimouse [Pierce Biotechnology, Rockford, IL, U.S.A.]). The immunoreactive bands were visualized using Super Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) and then measured using LAS-4000 mini (Fuji Film, Tokyo, Japan).

**Animal** The 6-week-old male ICR mice were used in these experiments. The mice were housed at 24±2°C under a 12 h light–dark cycle (lights on from 8:00 to 20:00) and had ad libitum access to food and water. Behavioral experiments were performed between 10:00 and 18:00. All procedures relating to animal care and treatment conformed to the animal care guidelines of the Animal Experiment Committee of Gifu Pharmaceutical University. All efforts were made to minimize both suffering and the number of animals used.

**Drug Treatment** Luteolin (50 mg/kg) was suspended in the 0.5% carboxymethylcellulose (Wako) solution and per orally (p.o.) administered once a day for 23 d. The tail suspension test and forced swim test were performed on the 20th and 22nd d, respectively.

Imipramine hydrochloride (Wako) was dissolved in saline and orally (for chronic treatment, once a day) or intraperitoneally (for acute treatment) administered.

**Tail Suspension Test** Thirty minutes after drug treatment, the tails of the mice were suspended with an adhesive tape 50 cm above the floor, and their behavior was recorded for 7 min. As a parameter for the test, the total duration of immobility during the last 5 min of the test was measured manually in a blind manner.

**Forced Swim Test** Thirty minutes after drug treatment, mice were placed individually in a glass cylinder (height 22 cm, diameter 16 cm) filled with 14 cm deep water (23±1°C) for 6 min, and the total period of immobility was recorded during the last 4 min. Mice were judged to be immobile when they remained floating passively in the water, making only small movements to keep their heads above the water. Measurement of immobility time was performed in a blind manner.

**Chronic Corticosteroid Treatment** Corticosterone (25 μg/ml free base; 4-pregnen-11β,21-diol-3,20-dione-21-hemisuccinate; Steraloids, Inc., Newport, RI, U.S.A.) was prepared as described in a previous report. Group-housed mice were presented with corticosterone in place of normal drinking water for 14 d, resulting in a dose of approximately 8.7 mg/kg/d. Animals were weaned with 3 d of 12.5 μg/ml, then 3 d with 6.25 μg/ml, to allow for gradual recovery of endogenous corticosterone secretion. Luteolin (50 mg/kg/d) and imipramine (10 mg/kg/d) were orally administered once a day from 14 to 35 d. Brain sampling was performed at 35 d.

**Statistical Analysis** Data are presented as means±S.E.M. Statistical comparisons were made by the Student’s t-test or one-way analysis of variance (ANOVA) followed by Dunnett’s test using Statview version 5.0 (SAS Institute Inc., Cary, NC, U.S.A.), with p<0.05 considered to indicate statistical significance.

**RESULTS**

**Effect of Luteolin against Tunicamycin-Induced SH-SY5Y Cell Death** The chemical structural formula is shown in Fig. 1A. We first examined whether luteolin protects against the neuronal cell death induced by tunicamycin in SH-SY5Y cells. Typical photographs of Hoechst 33342 and propidium iodide staining are shown in Fig. 1B. By comparison with the control group, tunicamycin at 2.0 μg/ml treatment significantly increased the number of dead cells (stained with propidium iodide). Pretreatment with luteolin at 0.1 to 10 μM protected against this cell death in a concentration-dependent manner and the effect was significant at 1 and 10 μM. To confirm whether the protective effect of luteolin was caused by its antioxidant activity, we evaluated the effects of a vitamin E analogue, Trolox, and N-acetylcysteine (NAC). However, neither Trolox at 10 μM nor NAC at 1 μM exhibited a protective effect against the tunicamycin-induced cell death (Fig. 1C).

**Effects of Luteolin on the Tunicamycin-Induced Increase in GRP78 and GRP94 Expression and the Cleavage Activation of Caspase-3 in SH-SY5Y Cells** To clarify the mechanism of the inhibitory effect of luteolin against endoplasmic reticulum stress-induced neuronal cell death, we performed Western blot analysis using SH-SY5Y cells. First, to examine whether luteolin would affect the endoplasmic
reticulum stress pathway, we measured GRP78 and GRP94 expressions (Fig. 2A). Tunicamycin remarkably upregulated the expressions of GRP78 and GRP94 (vs. control); however luteolin did not affect the level of GRP78 or GRP94 expression induced by tunicamycin. On the other hand, luteolin significantly induced GRP78 and GRP94 expression by itself (Figs. 2B, C). To confirm whether luteolin affects other unfolded protein response-induced proteins, we measured CHOP expression. Tunicamycin increased the band intensity of CHOP, although luteolin at 10 μM did not affect the expression of CHOP (vs. vehicle). Data represent means and S.E.M., n=6. #p<0.05 or ##p<0.01 vs. Cont (Control). (D) Expression of CHOP was detected using Western blotting. Luteolin at 10 μM or tunicamycin at 2.0 μg/ml was added and followed by 24h of additional incubation. (E) Expression of CHOP was quantified as described below. Data represent means and S.E.M., n=3. *p<0.05 vs. Cont (Control). (F) Expression of cleaved caspase-3 was detected using Western blotting. (G) Expression of cleaved caspase-3 was quantified by densitometry and corrected by reference to β-actin. Data represent means and S.E.M., n=5 or 6. **p<0.01 vs. Veh (Vehicle; tunicamycin-treated group).

Effect of Luteolin on Depression-Like Behaviors in the Tail Suspension and Forced Swim Tests We evaluated the effect of luteolin on depression-like behaviors using tail suspension and forced swim tests. These tests are commonly used to evaluate the antidepressant capacity. In these tests, we used immobility time as an indicator of depression. Since the acute or chronic treatment of general antidepressants specifically inhibit the immobility time in these tests, the utility of these experiments is recognized. In this time, we confirmed that imipramine (20 mg/kg, i.p.) significantly decreased immobility time in both tests (Figs. 3B, D). We performed tail suspension test and forced swim test on the 9th and 11th d, respectively, but there was no change in the immobility time between control and luteolin-treated group. In the tail suspension test, a chronic (for 20 d) luteolin (50 mg/kg/day (p.o.)) treatment decreased the despair state as assessed by immobility time (Fig. 3A). In the forced swim test,
chronic (for 22 d) luteolin-treated mice also showed a reduction of the despair state (Fig. 3C).

**Luteolin Regulates Expressions of GRP78 and GRP94 in the Hippocampus of Chronic Corticosterone Exposure Depression Model Mice**

To examine whether the antidepressant-like effects of luteolin might be exerted by regulating the expression of endoplasmic reticulum stress-related proteins, we used a corticosterone-treated depression model. The experiment protocol was designed by reference a previous report.6) We administered the luteolin for 21 d, because about 3-weeks-luteolin treatment showed antidepressant-like effect in normal mice (Figs. 3A, C). In the Western blot analysis using the hippocampus, expressions of GRP78 and GRP94 were significantly upregulated in corticosterone exposure mice (vs. control; Fig. 4). In luteolin (50 mg/kg/d, p.o.) and imipramine (10 mg/kg/d, p.o.) treatment groups, expressions of GRP78 and GRP94 were significantly reduced compared to the vehicle (Figs. 4B, C).

**DISCUSSION**

Flavonoids have a variety of pharmacological properties. It had been reported that a flavonoid, apigenin, which has the similar structure with luteolin, showed antidepressant-like effect18) and the protective effect against endoplasmic reticulum stress-induced neuronal cell death.19) Furthermore, chrysin also has the protective effect against endoplasmic stress-induced neuronal cell death.20) These findings indicate the possibility that luteolin has the protective effects against endoplasmic reticulum stress-induced depression and/or cell damage. In the present study, we investigated the effects of luteolin against endoplasmic reticulum stress-induced neuronal cell death in vitro and depression in vivo. Luteolin inhibited tunicamycin-induced cell death in SH-SY5Y cells. In vivo, luteolin showed an antidepressant-like effect.

It has been reported that exogenous stress induces neuronal cell death21,22) and decreases neurogenesis in the adult rat hippocampus.23) In postmortem brain studies, increased levels of endoplasmic reticulum stress-related proteins such as GRP78, GRP94, and calreticulin have been found in the temporal cortex of subjects with major depressive disorder who died of suicide.5) In corticosterone-treated animal depression models, increased levels of GRP78 and other endoplasmic reticulum stress-related proteins have been reported in the dentate gyrus and other region.6,24) These reports suggest that neuronal cell death and endoplasmic reticulum stress may be involved in the pathogenesis of depression.

In this study, we used tunicamycin, an endoplasmic reticulum stressor, to induce neuronal cell death in SH-SY5Y cells. Luteolin, but not Trolox or NAC, inhibited the tunicamycin-induced cell death in SH-SY5Y cells and our results suggest that the protective effect of luteolin against tunicamycin-induced cell death might be independent of its antioxidant activity.

Previously, it has been reported that luteolin increases the expression of GRP94 in both mRNA and protein levels, not
via the UPR signaling pathway in HeLa cells. In the present study, we examined the change of endoplasmic reticulum stress-related-protein expression using SH-SY5Y cells. Luteolin itself significantly increased the expressions of GRP78 and GRP94 without induction of CHOP protein; however, luteolin did not affect the level of GRP78 or GRP94 expression induced by tunicamycin. It has been reported that GRP78 has a protective effect on endoplasmic reticulum stress-induced cell death and that the compound which induces the expression of GRP78 inhibits the neuronal damage. GRP94 also has been reported to reduce the cell death in SH-SY5Y cells. These findings indicate that luteolin is not just an endoplasmic reticulum stressor such as tunicamycin, and may exert its protective effect by induction of molecular chaperones; however, the protective mechanism of luteolin is not direct action to molecular chaperones.

The caspase family is believed to play an important role in the effector phase of apoptosis in many paradigms. In an animal model of depression, caspase-3 positive cell was increased in the cerebral cortex, suggesting apoptosis. When the amount of unfolded protein exceeds a certain threshold, damaged cells are committed to cell death, which is mediated by the activation of caspase-3. In the present study, luteolin decreased in caspase-3 activation which was activated by tunicamycin treatment; luteolin also showed a protective effect against tunicamycin-induced cell death. As the effect of luteolin was small compared to protection against the tunicamycin-induced cell death, luteolin might also act on other pathways of the protective mechanism.

Next, we investigated the antidepressant effect of luteolin in vivo. Luteolin decreased the immobility time in the tail suspension test and the forced swim test. In the present study, we did not evaluate the effect of acute treatment of luteolin, because luteolin shows various effects by chronic treatment in previous reports. These results suggest that chronic treatment of luteolin has an antidepressant-like effect in the animal models. Previously, it had been reported that chronic luteolin (5 mg/kg, p.o.) treatment increased the immobility time in the forced swim test and showed anxiolytic effect in several behavioral tests. These finding indicate that low doses of luteolin show the anxiolytic effect, on the other hand, high doses of luteolin show the antidepressant-like effect.

Luteolin exists in a variety of forms, such as aglycon and glycoside forms, and produced and purified from the green peper leaf, which contains primarily the di-glycoside forms. Generally, it is known that glycoside forms are absorbed after they are converted to the form of aglycon. Thus, glycosides act as aglycons within organisms. Luteolin monoglycoside are also absorbed after converting to the aglycon form. In the present study, we also evaluated the effect of luteolin monoglycoside (50 mg/kg/d; luteolin aglycon equivalent, p.o.) in the tail suspension test. Luteolin monoglycoside decreased the immobility time in tail suspension test (data not shown). This suggests that luteolin monoglycoside also has an antidepressant-like effect in the animal model.

Corticosterone is an adrenal steroid hormone and secreted during stress, and it is necessary for the survival of the organism. It has been reported that chronic oral corticosterone exposure made mice depressed and increased the expression of endoplasmic reticulum stress-related proteins such as GRP78 and calreticulin in the dentate gyrus. Recently, we found that 6 h restraint stress increased the expression of endoplasmic reticulum stress-related genes in the mouse brain and plasma corticosterone levels. These findings indicate a possibility of a relationship among exogenous stress, endoplasmic reticulum stress, and corticosterone.

In the present study, corticosterone significantly increased in the expression level of GRP78 and GRP94 in the hippocampus; following this, luteolin and imipramine treatments reversed the expressions of GRP78 and GRP94. Luteolin monoglycoside (50 mg/kg/d; luteolin aglycon equivalent, p.o.) also decreased the expressions of GRP78 and GRP94 protein (date not shown). In an in vitro study, luteolin was administered before treatment with the endoplasmic reticulum stressor, tunicamycin, and Western blot analysis was performed after 24 h. Therefore, many unfolded proteins might still have existed in endoplasmic reticulum at the sampling, and the levels of GRP78 and GRP94 did not change with the luteolin treatment compared to the vehicle treatment. In an in vivo model, luteolin or imipramine was administered after the corticosterone exposure was performed, and Western blot analysis was carried out 21 d after the corticosterone treatment. Thus, the number of unfolded proteins present in endoplasmic reticulum decreased because of the induction of molecular chaperones, and the expressions of GRP78 and GRP94 might have decreased at the sampling point. Recently, the involvement of GRP94 in the treatment for bipolar disorder had been reported. These findings indicate that the antidepressant-like effects of luteolin could be due to the inhibition of the endoplasmic reticulum stress in the pre-induction of GRP78 and GRP94. However, further experiments will be needed to clarify the detailed mechanism.

In conclusion, these findings indicate that luteolin has an inhibitory activity on endoplasmic reticulum stress-induced cell death, in a part, through the induction of molecular chaperones. This effect may contribute the antidepressant-like effect of luteolin and help in the prevention of the depression.

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