Flavonoids Suppress the Cytotoxicity of Linoleic Acid Hydroperoxide toward PC12 Cells

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The suppressive effect of flavonoids on the cytotoxicity of linoleic acid hydroperoxide (LOOH) toward rat pheno
cromocytoma PC12 cells was examined. The extent of cytotoxicity was shown on the basis of % survival

determined by the trypsin blue exclusion test. On preincubation of cells with either 3-hydroxyflavone, quercetin,
or luteolin prior to LOOH exposure, the cytotoxicity was considerably suppressed. In contrast, on coincubation

of cells with either eriodictyol, quercetin, kaempherol, luteolin, or 3-hydroxyflavone and LOOH, it was markedly

suppressed. Regardless of incubation conditions, quercetin, 3-hydroxyflavone, and luteolin were thus more effec
tive as protective agents against the cytotoxicity than the other flavonoids. These flavonoids further showed a sup-

pressive effect on coincubation rather than on preincubation. These results suggest that such flavonoids are benefi
cial for cells under oxidative stress.

MATERIALS AND METHODS

Materials LOOH [(9Z,11E)-13-hydroperoxy-9,11-octadecadienoic acid] was prepared from linoleic acid with soy-
bean lipoxynase as described previously. Apigenin, 3-
hydroxyflavone, kaempherol, luteolin, naringenin, quercetin,
rutin, and DL-α-tocopherol were purchased from Wako Pure
Chemical Industries, Ltd. (Osaka, Japan), eriodictyol and

taxifolin from Extrasynthese S. A. (Genay, France), cyclo-

deheximide and adenosine 9-β-D-arabinofuranoside from Sigma-
Aldrich Japan K. K. (Tokyo, Japan), and the Determiner LPO

kit from Kyowa Medics Co., Ltd. (Tokyo). The RPMI1640

medium was obtained from Nissui Pharmaceutical Co., Ltd.
(Tokyo), and fetal bovine serum (FBS) and horse serum from

Moregate Laboratories (Melbourne, Australia). The horse

serum was inactivated at 56 °C for 30 min. PC12 cells came

from the Japanese Health Sciences Foundation (Osaka). Fal-

con plastic plates were purchased from Becton Dickinson

Labware (Lincoln Park, NJ, U.S.A.). Other materials were

obtained from ordinary commercial sources.

Cell Culture Cell cultivation was conducted at 37 °C in a

humidified atmosphere of 5% carbon dioxide and 95% air.
Cell counting was done with a Coulter counter (Model

Z-1, Coulter Corporation, Hialeah, FL, U.S.A.). Cells were incul

ated at a density of 2 × 10⁴ cells·cm⁻² in 10-cm plastic
plates, each of which contained 8 ml of the RPMI1640

medium supplemented with 5% FBS and 10% horse serum,

and cultivated. They were subcultivated every 3 d at a 3 : 1

split ratio by pipetting. The medium was changed 2 d after

inoculation.

Quantification of the Suppressive Effects of Flavonoids on the Cytotoxicity of Linoleic Acid Hydroperoxide

Each flavonoid or α-tocopherol was solubilized in ethanol at a concentration of 25 mM, 2 μl of which was administered to
cells in 1 ml of Earle's solution: i.e., 50 μM each in the 0.2% ethanol-containing solution. Less than 0.5% of ethanol at the
final concentration had no effect on cells.

Cells were inoculated at a density of 5 × 10⁴ cells·cm⁻² in 24-well plastic plates, each well of which contained 1 ml of the
FBS and horse serum-supplemented medium, and cultured overnight.

For preincubation with each flavonoid prior to LOOH ex-
posure, the medium was removed and cells in each well were incubated with 50 μM of either a flavonoid or α-tocopherol in 1
ml of the FBS and horse serum-supplemented medium for an appropriate period of time. After removal of the medium,
the cells were washed with 1 ml of calcium and magnesium-
free phosphate-buffered saline at pH 7.4 (PBS) and incubated
for 1.5 h in 1 ml of Earle's solution containing 50 μM LOOH.

For coincubation with each flavonoid and LOOH, the medium was removed and cells in each well were washed with
1 ml of PBS. They were incubated for 1.5 h in 1 ml of Earle's solution containing 50 μM of either a flavonoid or α-
tocopherol and 50 μM LOOH.

Key words flavonoid; linoleic acid hydroperoxide; PC12 cell; cytotoxicity; antioxidant activity

Flavonoids are a group of plant polyphenols which are found in abundance in food and drinks and are consumed. They have recently attracted much attention because of their broad pharmacological activities, in particular antioxidant activities. Reactive oxygen species-induced oxidative stress is suggested to play important roles in the etiology of neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, which are intimately associated with neuronal death. If the oxidative stress is suppressed by dietary factors such as flavonoids, this group may serve as a preventive agent against the cytotoxicity of the other flavonoids. These flavonoids further showed a suppressive effect on coincubation rather than on preincubation. These results suggest that such flavonoids are beneficial for cells under oxidative stress.

Materials and Methods

Materials LOOH [(9Z,11E)-13-hydroperoxy-9,11-octadecadienoic acid] was prepared from linoleic acid with soybean lipoxynase as described previously. Apigenin, 3-hydroxyflavone, kaempherol, luteolin, naringenin, quercetin, rutin, and DL-α-tocopherol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), eriodictyol and taxifolin from Extrasynthese S. A. (Genay, France), cycloheximide and adenosine 9-β-D-arabinofuranoside from Sigma-Aldrich Japan K. K. (Tokyo, Japan), and the Determiner LPO kit from Kyowa Medics Co., Ltd. (Tokyo). The RPMI1640 medium was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo), and fetal bovine serum (FBS) and horse serum from Moregate Laboratories (Melbourne, Australia). The horse serum was inactivated at 56 °C for 30 min. PC12 cells came from the Japanese Health Sciences Foundation (Osaka). Falcon plastic plates were purchased from Becton Dickinson Labware (Lincoln Park, NJ, U.S.A.). Other materials were obtained from ordinary commercial sources.

Cell Culture Cell cultivation was conducted at 37 °C in a humidified atmosphere of 5% carbon dioxide and 95% air. Cell counting was done with a Coulter counter (Model Z-1, Coulter Corporation, Hialeah, FL, U.S.A.). Cells were inoculated at a density of 2 × 10⁴ cells·cm⁻² in 10-cm plastic plates, each of which contained 8 ml of the RPMI1640 medium supplemented with 5% FBS and 10% horse serum, and cultivated. They were subcultivated every 3 d at a 3 : 1 split ratio by pipetting. The medium was changed 2 d after inoculation.

Quantification of the Suppressive Effects of Flavonoids on the Cytotoxicity of Linoleic Acid Hydroperoxide

Each flavonoid or α-tocopherol was solubilized in ethanol at a concentration of 25 mM, 2 μl of which was administered to cells in 1 ml of Earle's solution: i.e., 50 μM each in the 0.2% ethanol-containing solution. Less than 0.5% of ethanol at the final concentration had no effect on cells.

Cells were inoculated at a density of 5 × 10⁴ cells·cm⁻² in 24-well plastic plates, each well of which contained 1 ml of the FBS and horse serum-supplemented medium, and cultivated overnight.

For preincubation with each flavonoid prior to LOOH exposure, the medium was removed and cells in each well were incubated with 50 μM of either a flavonoid or α-tocopherol in 1 ml of the FBS and horse serum-supplemented medium for an appropriate period of time. After removal of the medium, the cells were washed with 1 ml of calcium and magnesium-free phosphate-buffered saline at pH 7.4 (PBS) and incubated for 1.5 h in 1 ml of Earle's solution containing 50 μM LOOH.

For coincubation with each flavonoid and LOOH, the medium was removed and cells in each well were washed with 1 ml of PBS. They were incubated for 1.5 h in 1 ml of Earle's solution containing 50 μM of either a flavonoid or α-tocopherol and 50 μM LOOH.
The % survival of cells was determined by the trypan blue exclusion test. Using the lipid peroxide quantification kit “Determiner LPO”, it was confirmed that none of the flavonoids reacted with LOOH in Earle’s solution within at least 1.5 h.

**The Effect of Cycloheximide or Adenine 9-β-d-Arabinofuranoside on the % Survival of Quercetin-Preincubated, Linoleic Acid Hydroperoxide-Incubated Cells**

Cells were inoculated at a density of $5 \times 10^4$ cells·cm$^{-2}$ in 24-well plastic plates, each well of which contained 1 ml of the FBS and horse serum-supplemented medium, and cultivated overnight. After removal of the medium, cells in each well were incubated with 50 μM of quercetin and either 4 μM cycloheximide or 10 μM adenine 9-β-d-arabinofuranoside in 1 ml of the FBS and horse serum-supplemented medium for 24 h. After removal of the medium, they were washed with 1 ml of PBS and incubated for 1.5 h in 1 ml of Earle’s solution containing 50 μM LOOH.

**Statistical Analysis**

Data are expressed as means and standard deviations. Differences in the mean values were assessed for significance by one-way analysis of variance (ANOVA) and Fisher’s PLSD; $p$-values $<0.05$ were considered significant. Statistical analysis was performed using a Macintosh G4 computer (Apple Computer, Inc., Cupertino, CA, U.S.A.) with Stat View-J 5.0 software (Abacus Concepts, Inc., Berkeley, CA, U.S.A.).

**RESULTS AND DISCUSSION**

Figure 1 shows that LOOH is cytotoxic toward PC12 cells. Their % survival was 14.4±4.7%, when they were exposed to 50 μM LOOH in Earle’s solution for 1.5 h. The suppressive effect of flavonoids on LOOH cytotoxicity was examined. Two flavones of apigenin and luteolin, three flavonols of 3-hydroxyflavone, kaempferol, and quercetin, a flavanol glycoside of rutin, and two flavanones of naringenin and eriodictyol, and a flavanol of taxifolin were used, the chemical structures of which are depicted in Table 1. α-Tocopherol, which is a typical chain-breaking antioxidant, was used as a standard antioxidant. The suppressive effect of the flavonoids was compared with that of α-tocopherol. In addition, it was confirmed using the Determiner LPO kit that none of them reacted with LOOH in Earle’s solution within at least 1.5 h (data not shown).

Figure 1 shows the suppressive effect of preincubated flavonoids on LOOH cytotoxicity toward PC12 cells. On preincubation of cells with 50 μM of each flavonoid prior to 50 μM-LOOH exposure, 3-hydroxyflavone, quercetin, and luteolin had similar suppressive effects to that of α-tocopherol, and taxifolin had a somewhat suppressive effect. Apigenin, kaempferol, rutin, and naringenin, and eriodictyol had no effect.

Figure 2 shows the suppressive effect of coincubated flavonoids on LOOH cytotoxicity toward PC12 cells. On coincubation of cells with 50 μM of each flavonoid and 50 μM LOOH, eriodictyol, quercetin, kaempferol, luteolin, and 3-hydroxyflavone had a more suppressive effect than α-tocopherol, while that of taxifolin was similar to α-tocopherol. Apigenin, naringenin, and rutin had no effect.

As shown in Figs. 1 and 2, these flavonoids were effective on coincubation rather than on preincubation. On preincubation, 3-hydroxyflavone, quercetin, and luteolin were more effective than the other flavonoids, and on coincubation, these three flavonoids, eriodictyol, and kaempferol were also more effective. Interestingly, eriodictyol, kaempferol, and taxifolin had a protective effect only on coincubation, although the reason is unknown. Both on preincubation and coincubation, the flavone apigenin, the flavanone naringenin, and the flavonol glycoside rutin had no effect. These results suggest that either the 3-hydroxy group or the 3’4’-diol groups are necessary for the suppressive effect. Rutin may be ineffective due to its low lipophilicity.

Figure 3 shows the time course of the suppressive effect of preincubated quercetin on LOOH cytotoxicity. The effect was enhanced up to 3 h in a preincubation time-dependent manner and thereafter remained unchanged. This suggests that quercetin is incorporated into cells, at least plasma membranes, and suppresses LOOH cytotoxicity. Presumably, 3-
hydroxyflavone and luteolin, as well as quercetin, are incorporated into cells also.

It has been suggested that flavonoids may affect gene expression in cells; e.g., quercetin and apigenin may act as inhibitors of cytokine-induced gene expression. However, inclusion of 4 μM cycloheximide, an inhibitor of protein synthesis, or 10 μM adenine 9-β-D-arabinofuranoside, an inhibitor of DNA synthesis, into preincubation medium containing 50 μM quercetin had no effect on cell survival (data not shown). This implies that the suppressive effect of preincubated quercetin is not related to gene expression.

On the basis of the Trolox equivalent antioxidant activity, the antioxidant activity of the above flavonoids in homogeneous solution is in the following order: quercetin > rutin > luteolin > taxifolin > kaempherol > naringenin > apigenin. This shows that for the antioxidant activity, the 5,7,3′,4′-tetrahydroxyl system, quercetin, rutin, luteolin, and taxifolin, is more important than the 5,7,4′-trihydroxyl system, kaempferol, naringenin, and apigenin, and the 3-hydroxyl group is unnecessary as deduced from the high activity of rutin, whose 3-hydroxyl group is blocked by linkage with rutinoside. In homogeneous solution, thus, the structure–activity relationships of flavonoids appear to depend on the number of hydroxyl groups on their A and B rings and not to depend on the difference in their class of flavone, flavonol, flavanone, and flavanol.

It turns out that the suppressive effect of flavonoids on oxidative stress-induced cytotoxicity is very different from their antioxidant activity in homogeneous solution. Presumably, the suppressive effect may depend not only on their antioxidant activity but also on their affinity with and permeability through cell membranes.

On the other hand, we have reported that on coincubation, some flavonoids suppress LOOH cytotoxicity toward human umbilical vein endothelial cells. Their efficacy is in the following order: quercetin > luteolin > kaempherol > 3-hydroxyflavone. Apigenin, rutin, naringenin, and taxifolin are ineffective. On preincubation, further, all of these flavonoids are ineffective. Nègre-Salvayre and Salvayre reported that on both preincubation and coinucubation, quercetin and rutin suppressed the cytotoxicity of oxidized low density lipoprotein toward human lymphoid cell lines, and the former was much more effective than the latter. These observations suggest that the suppressive effect of flavonoids on oxidative stress-induced cytotoxicity differs depending on cell type.

Ishige et al. reported that several flavonoids, including quercetin, luteolin, kaempferol, galangin, and fisetin, suppressed oxidative stress-induced cytotoxicity toward the mouse HT-22 hippocampal cell line and rat primary neurons. When oxidative stress was induced with glutamate, the flavonoids suppressed the oxidative stress-mediated increases in reduced glutathione concentration, reactive oxygen species level, and calcium(II) ion influx within cells. In addition, it has been reported that on both coincubation and preincubation, some flavonoids protected chick retinal cells from ascorbate/iron(II)-induced lipid peroxidation and their efficacy was in the following order: eriodictyol > quercetin > luteolin > taxifolin, and that quercetin decreased the reactive oxygen species level within HL-60 cells and prevented dehydroascorbate-induced glutathione depletion in rabbit red blood cells. Although the suppressive mechanism of flavonoids against oxidative stress-induced cytotoxicity is not fully understood, their suppressive effect may be intimately related to a decrease in the intracellular generation of reactive oxygen species.

In conclusion, such flavonoids as quercetin, 3-hydroxyflavone, and luteolin may be superior to α-tocopherol as nutritional factors against oxidative stress, and the adequate intake of the flavonoids may be beneficial for cells under oxidative stress.

REFERENCES
4) Cohen G., "Reactive Oxygen Species in Biological Systems: An Inter-