Effects of Uptake of Flavonoids on Oxidative Stress Induced by Hydrogen Peroxide in Human Intestinal Caco-2 Cells

Atsushi YOKOMIZO and Masamitsu MORIWAKI

Fundamental Research Division, San-Ei Gen F.F.I. Corporation, 1-1-11 Sanwacho, Toyonaka, Osaka 561-8588, Japan

Received November 7, 2005; Accepted February 25, 2006; Online Publication, June 23, 2006

The relation between the uptake of flavonoids and the response of human colon adenocarcinoma Caco-2 cells exposed to oxidative stress induced by hydrogen peroxide (H₂O₂) was examined. Flavonoid aglycones were incorporated into Caco-2 cells in a concentration- and time-dependent manner, but neither glycosides nor unstable myricetin were incorporated into the cells. The incorporated flavonoids reduced the reactive oxygen species (ROS) induced by H₂O₂ in the cells in proportion to the amount incorporated and the radical scavenging activity of flavonoids. But, flavonoids with high radical scavenging activity also generated H₂O₂. Therefore, the decrease in the amount of intracellular ROS was inversely related to the H₂O₂ scavenging activity of flavonoids. These results suggest that strong antioxidant flavonoids have both a cytoprotective effect owing to the scavenging of ROS and cytotoxic effect caused by the generation of H₂O₂.

Key words: flavonoid; antioxidative activity; oxidative stress; hydrogen peroxide; Caco-2 cells

With the recent increase in lifestyle-related diseases, there is growing concern over their prevention by improving the diet. Over 4,000 species of flavonoids are widely present in plants, and they have been found to help prevent various diseases in humans.¹,² Flavonoids have antioxidative activity, affect the redox status, and can protect cells from oxidative stress. A recent animal study found that oral administration of flavonoids improved the resistance of plasma to oxidative stress.³ Studies using cells in culture have also demonstrated a cytoprotective effect of the incorporated flavonoids, due to suppression of oxidant-induced cell damage.⁴,⁵ Free-radical scavenging and metal-ion chelating activities have been proposed as the mechanism of the antioxidative activity of flavonoids.⁶ Previously we reported that myricitrin suppressed low-density lipoprotein (LDL) oxidation due to 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) radical scavenging and copper ion chelation.⁷ On the other hand, the pro-oxidative effects and cytotoxicity of flavonoids, possibly due to generation of ROS, including H₂O₂, and induction of apoptosis have been reported.⁸–¹⁰ Thus flavonoids appear to have both antioxidative and pro-oxidative effects, but clear evidence of prevention of oxidative stress by the antioxidative activity of flavonoids is lacking. Hence, we studied their activity using a cell culture system. In this study, we used human intestinal Caco-2 cells, which are frequently used to evaluate the bioavailability of bioactive substances such as food substances and drugs, due to their ability to differentiate into polarized enterocyte-like monolayers and the similarity of enzymes, transporters, and morphology to those of human intestinal epithelial cells.¹¹,¹²

The objective of our study was to elucidate the relation between the antioxidative activity of cellular incorporated flavonoids and their effect on the oxidative stress induced by H₂O₂, using human intestinal Caco-2 cells.

Materials and Methods

Materials. Myricitrin was obtained from Funakoshi (Tokyo), and other flavonoids were obtained from Sigma (St. Louis, MO). Figure 1 shows the structure of the individual flavonoids used in this study. A stock solution of flavonoids was prepared in dimethyl sulfoxide (DMSO) and diluted to give a final DMSO concentration not exceeding 0.5% in all treatment groups. Hydrogen peroxide was obtained from Wako Pure Chemicals.
Density of Caco-2 cells passage numbers 21 to 39 were seeded at a density of 10^5 cells/cm^2, and cultured to be confluent two times a week. The culture medium of confluent Caco-2 cell monolayers in 96-well plates were removed and rinsed, and oxidative stress was measured at 734 nm. The ABTS radical scavenging activity was represented in the dark. The decolorization ratio of DPPH was measured at 517 nm, and the DPPH radical scavenging activity was represented as ascorbic acid equivalent.

**Cell culture.** The human colon adenocarcinoma cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD). All cell culture media were obtained from Invitrogen (Carlsbad, CA). Caco-2 cells passage numbers 21 to 39 were seeded at a density of 5 × 10^5 cells/cm^2, and cultured to be confluent for 15–19 d postseeding in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). The cells were rinsed, and 1 ml was added to each well. After 15 to 120 min incubation in a CO_2 incubator, the medium of confluent Caco-2 cell monolayers in 96-well plates was removed and rinsed, and 1 ml was added to each well. After 15 to 120 min incubation in a CO_2 incubator, the medium was changed two times a week.

**Cellular uptake of flavonoids.** The culture medium of confluent Caco-2 cell monolayers in 24-well plates was removed and rinsed with medium for flavonoid treatment. Then flavonoids in DMSO solution were diluted to 0.05% TFA in methanol. The flow rate was 0.8 ml/min, and the linear gradient of phase B changed from 0 to 100% in 30 min. The eluate was monitored with a diode array detector with a detection limit of 0.1 μM.

**DPPH radical scavenging activity.** 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was evaluated according to the method of Blois. Flavonoids in a methanol solution were added to the DPPH methanol solution to make the final DPPH solution 100 μM, mixed vigorously and kept for 30 min at 37°C in the dark. The decolorization ratio of DPPH was measured at 517 nm, and the DPPH radical scavenging activity was represented as ascorbic acid equivalent.

**ABTS radical scavenging activity.** 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) dianionium salt (ABTS) radical scavenging activity was evaluated according to the method of Re et al. The ABTS radical cation (ABTS⁺) was produced by reacting 7 mM ABTS with 2.45 mM potassium persulfate in the dark at room temperature for 16 h. The ABTS⁺ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. Twenty microliters of flavonoids in an ethanol solution was added to the 180 μl of the ABTS⁺ solution. The mixture was kept for 6 min at room temperature, and measured at 734 nm. The ABTS⁺ radical scavenging activity was represented as ascorbic acid equivalent.

**Intracellular reactive oxygen species.** Generation of intracellular ROS was evaluated by intracellular oxidation of 2’,7’-dichlorofluorescin (DCFH) to the fluorescent compound 2’,7’-dichlorofluorescein (DCF). The culture medium of confluent Caco-2 cell monolayers in 96-well plates was removed and rinsed, and 1 to 50 μM flavonoids in HBSS were incubated for 1 h at 37°C. After treatment with flavonoids, the cells were rinsed and incubated for 30 min after the addition of 200 μl of 100 μM DCFH-DA in HBSS containing 1% FBS. The cells were rinsed, and 100 μl of 50 μM H_2O_2 was added and incubated another 1 h. Fluorescent generation was measured with a 1420 ARVOx Multi-label counter (Perkin Elmer, Wellesley, MA). The values are expressed as percentages of DCF fluorescence intensity to that of control cells without flavonoid treatment.

**LDH assay.** Intracellular enzyme lactate dehydrogenase (LDH) is released into the medium from the damaged cell. LDH activity was evaluated using the LDH Cytotoxic Test Wako (Wako Pure Chemicals). The confluent Caco-2 cell monolayers in 96-well plates were treated with 1 to 50 μM flavonoids in HBSS or DMEM for 1 h, the cells were rinsed, and oxidative stress was induced by 20 h incubation in 0.1 to 10 mM H_2O_2. Then 50 μl of the medium was transferred into a new well plate, and the amount of LDH was enzymatically determined according to the manufacturer’s protocol.

---

**Table 1. Structure of Flavonoids Studied.**

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myricitrin</td>
<td>O-α-Rha</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Rutin</td>
<td>O-β-Rut</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Isoquercitrin</td>
<td>O-β-Glc</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Quercetin</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Myricetin</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Luteolin</td>
<td>H</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Apigenin</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
</tbody>
</table>
**MTT assay.** Cell viability was evaluated by colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The confluent Caco-2 cell monolayers in the 96-well plates were treated with 1 to 50 μM flavonoids in HBSS or DMEM for 1 h, the cells were rinsed, and oxidative stress was induced by incubation in 0.1 to 10 mM H₂O₂ for 20 h. Then 20 μl of the MTT solution (5 mg/ml in HBSS) was added to each well, and the plate was incubated for another 4 h in a CO₂ incubator. The MTT formazan formed was solubilized by the addition of 100 μl of isopropanol containing 40 mM HCl. The absorbance of each sample was analyzed at 560 nm.

**Measurement of hydrogen peroxide.** Hydrogen peroxide concentrations were quantified by the ferrous ion oxidation-xylenol orange method (FOX assay), using Bioxytech H₂O₂-560 (Oxis International, Portland, OR). For the H₂O₂ generation study, 50 μM of flavonoids was incubated in HBSS with or without 100 units/ml of catalase. For the study of H₂O₂ scavenging, 50 μM H₂O₂ and 50 μM flavonoids were co-incubated in HBSS at 37 °C. The hydrogen peroxide concentrations of these solutions were measured periodically.

**Statistical analysis.** Each data point represents the mean of replicated samples, and the result is expressed as the mean ± SD. Differences between means were compared using one-way and two-way ANOVA for repeated measurements. A post-hoc analysis was done using the Tukey-Kramer HSD test. A value of p < 0.05 was judged to be statistically significant.

**Results and Discussion**

**Uptake of flavonoids**

The degree of cellular uptake of various flavonoids was investigated by incubation with Caco-2 cell monolayers in HBSS or DMEM (Fig. 2). The flavonoid uptake in HBSS medium was 2- to 4-fold that in DMEM medium. After a 2-h incubation in 50 μM flavonoid solution, the order of cellular uptake was: quercetin > kaempferol > luteolin > apigenin in HBSS, kaempferol > luteolin > apigenin > quercetin in DMEM. The degree of cellular uptake of flavonoids differed with the kind of medium. Although flavonoid aglycones were incorporated into Caco-2 cells, neither myricetin nor any glycosides were incorporated from these media. This difference in cellular uptake between aglycones and glycosides perhaps derived from the lipophilicity of the flavonoids. This suggests that flavonoid glycosides must be metabolized to be well absorbed. Human and animal studies have shown that flavonoid glycosides are absorbed after metabolism, such as deglycosylation and glucuronidation. The reason myricetin, an aglycone, was not incorporated might have been its instability under this experimental condition. Analysis by HPLC revealed that myricetin was rapidly degraded just after addition to either medium (Table 1).

The effects of incubation time and concentration on the manner of uptake of flavonoids were investigated in HBSS and DMEM (Fig. 3). In both media, the flavonoids were incorporated in a dose-dependent manner up to 50 μM. No significant cytotoxic effects of flavonoids were observed under this experimental condition. The flavonoids were rapidly incorporated for 30 min after the start of incubation, and thereafter were incorporated slowly. These results suggest that the appropriate condition for treatment with flavonoids in this system is a flavonoid concentration of 50 μM and an incubation time of 60 min.

Flavonoid distribution and total recovery were investigated after incubation with 50 μM of flavonoid in HBSS (Table 1). Glycosides were not incorporated, and were recovered only from the medium fraction, whereas...
aglycones were recovered from both cellular extracts and medium fractions. As for unstable myricetin, degraded and trace amounts were observed in the medium fractions. In the total recovery ratio, all added glycosides were recovered, but the recovery rate of aglycones was 73 to 92%. The specific binding with the cell components and metabolism might be responsible for the decrease in total recovery of the aglycones. Myricitrin, myricetin, and quercetin had strong radical scavenging activity, although apigenin had no activity against either radical. Hydroxyl groups have been proposed to be important for antioxidative potential.22) Walle et al. reported that covalent binding of quercetin to DNA and protein was observed by incubation with Caco-2 cells.20) Murota et al. reported that the flavonoids aglycone, quercetin, kaempferol, luteolin, and apigenin were incorporated into Caco-2 cells, and converted to their glucuronide and sulfate.21)

**Measurement of radical scavenging activity**

The radical scavenging activity of flavonoids was measured using the DPPH and ABTS radical (Table 2). Myricitrin, myricetin, and quercetin had strong radical scavenging activity, although apigenin had no activity against either radical. Hydroxyl groups have been proposed to be important for antioxidative potential.22)

**Effects of oxidative stress**

Cellular flavonoids have been shown to protect the cell by preventing oxidative cell damage induced by H\textsubscript{2}O\textsubscript{2}, xanthine oxidase, and metal ions.4,23) Peng and Kuo reported that pre-incubation of Caco-2 cells with quercetin and luteolin suppressed malondialdehyde formation and reduced the oxidative stress induced by H\textsubscript{2}O\textsubscript{2} and iron.24) To examine the relation between the radical scavenging activity of incorporated flavonoids and their cytoprotective effect on the oxidative stress induced by H\textsubscript{2}O\textsubscript{2}, we quantified intracellular ROS by DCFH assay.

We used HBSS as the medium, because rapid decomposition of H\textsubscript{2}O\textsubscript{2} was observed in DMEM at 37°C, but not in HBSS (Fig. 4A). After incubation of 50µM H\textsubscript{2}O\textsubscript{2} for 30 min in DMEM, the amount of H\textsubscript{2}O\textsubscript{2} remaining was less than 1 µM. This decomposition has
Effects of Flavonoids on Oxidative Stress in Caco-2 Cells

Fig. 4. Effect of Culture Medium Species and Caco-2 Cells on H₂O₂ Concentration.
Fifty μM of H₂O₂ was incubated in four kinds of cell culture medium without (A) or with (B) the Caco-2 cell monolayer. Hydrogen peroxide concentrations of cell culture medium were measured with time by the ferrous ion oxidation-xylenol orange method. ●, HBSS; ○, HBSS containing 1% FBS; ■, DMEM; □, DMEM containing 10% FBS. Each data point shows the mean ± SD (n = 6).

also been reported in other culture media, and the factor is not clear.25) When the Caco-2 cell monolayer was incubated with H₂O₂ in HBSS, H₂O₂ was gradually incorporated into the cells (Fig. 4B).

After exposure of the Caco-2 cell monolayer to H₂O₂, the amount of intercellular ROS increased concentration dependently, the amount after treatment with 50 μM of H₂O₂ being 3- to 5-fold that without H₂O₂ treatment. Pre-incubation with flavonoids that could be incorporated into the cells, viz., quercetin, kaempferol, and luteolin, significantly decreased the amount of intracellular ROS induced by H₂O₂, but pre-incubation with apigenin and the other flavonoids that could not be incorporated into the cells did not (Fig. 5). The absence of decrease with apigenin was due to its weak radical-scavenging activity. The ability to be incorporated into the cell was requisite to decrease intracellular ROS. The residual amount of intracellular ROS after H₂O₂ treatment was quercetin (45.7%) < luteolin (65.3%) = kaempferol (74.1%) < apigenin (116.4%). This effect was correlated with the radical scavenging activity. These results suggest that radical scavenging activity was important in decreasing intracellular ROS.

The effects of flavonoid concentration on the decrease in ROS were also investigated (Fig. 6). The concentration at which a significant decrease in ROS induced by 50 μM H₂O₂ was observed was 2 to 50 μM for quercetin, 20 to 50 μM for kaempferol, and 10 to 50 μM for luteolin. These results suggest that not only the incorporation of flavonoids that have radical scavenging activity, but also the degree of incorporation was important in decreasing intracellular ROS.

By measurement of intracellular LDH leakage and MTT reduction assay, we evaluated the effect of flavonoids on the Caco-2 cell monolayer exposed to oxidative stress induced by H₂O₂. The results of both methods indicated that exposure of Caco-2 cells to 0.3 to 10 mM H₂O₂ showed significant damage depending on the concentration. Pre-incubation with 5 to 50 μM flavonoids before H₂O₂ treatment did not show any cytoprotective effect (data not shown). These results indicate that the flavonoids must be incubated with H₂O₂ for measurement of the cytoprotective effect.

Relation to hydrogen peroxide

To determine the relation between flavonoid and H₂O₂, we measured the generation and scavenging
Fig. 6. Effect of Flavonoid Concentration on H$_2$O$_2$ Induced Intracellular ROS Generation.

Caco-2 cell monolayers were incubated with 1 to 50 $\mu$m flavonoids for 1 h in HBSS, and after removal of flavonoid solution, incubated with 100 $\mu$m DCFH-DA for 30 min, and then with 50 $\mu$m H$_2$O$_2$ for 1 h. Then fluorescence was measured. The values are expressed as percentages of the DCF fluorescence intensity to that of control cells incubated without flavonoids. Each data point shows the mean $\pm$ SD (n = 8–9). *Significant difference from the control ($p < 0.05$).

ability of H$_2$O$_2$ (Table 3). H$_2$O$_2$ generation was investigated by incubation of 50 $\mu$m flavonoid for 2 h in HBSS. Flavonoids that have strong radical scavenging activity, viz., myricitrin, quercetin, and myricetin, generated H$_2$O$_2$ markedly during 2 h incubation in HBSS, but, none of the other flavonoids showed any significant generation of H$_2$O$_2$. The H$_2$O$_2$ generation was completely eliminated by the addition of catalase. The generation of H$_2$O$_2$ from flavonoids was perhaps derived from the strong hydrogen donating ability to oxygen. Miura et al. also reported that 7 out of 14 flavonoids generated H$_2$O$_2$ by incubation in acetate buffer, and suggested that pyrogallol or catechol structure was involved in generation.$^{10}$ The activity of H$_2$O$_2$ scavenging was investigated by co-incubation with 50 $\mu$m flavonoid and 50 $\mu$m H$_2$O$_2$ for 2 h in HBSS. Myricetin slightly increased H$_2$O$_2$ over the added amount, and all other the flavonoids were reduced. Interestingly, apigenin had no activity as to radical scavenging or decreasing intracellular ROS, although it showed a decrease in H$_2$O$_2$. The order of H$_2$O$_2$ scavenging activity after incubation for 2 h was in the following order: luteolin = kaempferol = apigenin = rutin = isoquercitrin > quercetin = myricitrin > myricetin. When flavonoids were co-incubated with the Caco-2 cell monolayer, the same order of H$_2$O$_2$ generation and scavenging ability was observed (data not shown). The activity of H$_2$O$_2$ scavenging was inversely related to the decrease in intracellular ROS and radical scavenging activity. Hence, it was suggested that the mechanism of decrease of intracellular ROS induced by H$_2$O$_2$ was not due to direct H$_2$O$_2$ scavenging, but rather to scavenging of ROS generated from H$_2$O$_2$.

Time-course generation and scavenging of H$_2$O$_2$ was also investigated (Fig. 7). Myricitrin, quercetin, and myricetin generated H$_2$O$_2$ markedly during the first 1 h of incubation. Especially rapid degradation of myricetin was observed within the first 15 min, and abundant H$_2$O$_2$ was generated by auto-oxidation. During co-incubation with 50 $\mu$m flavonoid and 50 $\mu$m H$_2$O$_2$, each flavonoid immediately scavenged 13 to 29 $\mu$m of H$_2$O$_2$, but regeneration of H$_2$O$_2$ was observed with increasing incubation time. Myricetin scavenged H$_2$O$_2$ immediately, but only after 15 min of incubation was the amount of H$_2$O$_2$ recovered equal to the amount of H$_2$O$_2$ added. These results suggest that flavonoids that have strong radical scavenging activity act as pro-oxidant by the generating effect of H$_2$O$_2$ in the extracellular system.

In order to investigate the effect of H$_2$O$_2$ generation of flavonoids on intracellular ROS generation, the DCFH-DA treated Caco-2 cell monolayer was incubated with the flavonoid solution for 1 h (Fig. 8). Although myricitrin, quercetin, and myricetin generated H$_2$O$_2$ during 2 h incubation in HBSS, intracellular ROS decreased. DCFH was easily oxidized by ROS, but reactivity with H$_2$O$_2$ was poor, and DCFH existed not only in the cytoplasm but also in the cell membrane due to lipophilicity.$^{26}$ Perhaps these flavonoids inhibited the

### Table 3. Hydrogen Peroxide Generation and Scavenging by Flavonoids

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>H$_2$O$_2$ concentration ($\mu$m)</th>
<th>Generation</th>
<th>Generation with catalase</th>
<th>Residual amount of 50 $\mu$m H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myricitrin</td>
<td>5.4 ± 0.3$^b$</td>
<td>ND</td>
<td>37.1 ± 1.6$^b$</td>
<td></td>
</tr>
<tr>
<td>Rutin</td>
<td>ND</td>
<td>ND</td>
<td>27.0 ± 1.4$^b$</td>
<td></td>
</tr>
<tr>
<td>Isoquercitrin</td>
<td>ND</td>
<td>ND</td>
<td>27.9 ± 0.5$^b$</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>7.9 ± 0.5$^b$</td>
<td>ND</td>
<td>35.1 ± 1.1$^b$</td>
<td></td>
</tr>
<tr>
<td>Myricetin</td>
<td>27.6 ± 1.3$^b$</td>
<td>ND</td>
<td>52.1 ± 1.9$^b$</td>
<td></td>
</tr>
<tr>
<td>Kaempferol</td>
<td>ND</td>
<td>ND</td>
<td>26.3 ± 0.7$^b$</td>
<td></td>
</tr>
<tr>
<td>Luteolin</td>
<td>ND</td>
<td>ND</td>
<td>26.1 ± 0.4$^b$</td>
<td></td>
</tr>
<tr>
<td>Apigenin</td>
<td>ND</td>
<td>ND</td>
<td>26.8 ± 0.8$^b$</td>
<td></td>
</tr>
</tbody>
</table>

For the H$_2$O$_2$ generation study, 50 $\mu$m flavonoid solution was incubated in HBSS for 2 h with or without 100 unit/ml of catalase. For the H$_2$O$_2$ scavenging study, 50 $\mu$m H$_2$O$_2$ and 50 $\mu$m flavonoids were co-incubated in HBSS, and the residual amount of H$_2$O$_2$ was measured. The hydrogen peroxide concentrations of these solutions were measured by the ferrous ion oxidation-xylene orange method. Each data point shows the mean ± SD (n = 9–10). Values with different letters within a column are significantly different from each other ($p < 0.05$). ND, not detected.
DCFH auto-oxidation induced by ROS in the cell membrane. These results suggest that antioxidative flavonoids have a cytoprotective effect by scavenging ROS, although they have H$_2$O$_2$ generation ability.

In conclusion, flavonoids incorporated into the cells reduced the intracellular ROS induced by H$_2$O$_2$ in proportion to the amount incorporated and radical scavenging activity. The cytoprotective effect was not due to the direct scavenging of H$_2$O$_2$, but to the scavenging of ROS generated from H$_2$O$_2$. However, flavonoids with strong radical scavenging activity also showed the ability to generate H$_2$O$_2$. These results suggest that antioxidative flavonoids have both a cytoprotective effect due to scavenging ROS and a cytotoxic effect due to generation of H$_2$O$_2$. The antioxidative effect of flavonoids varies depending on the target ROS. Further studies are necessary to determine whether the flavonoids act to scavange ROS and generate H$_2$O$_2$ in vivo.

References


