Flavonoid Structure Affects the Inhibition of Lipid Peroxidation in Caco-2 Intestinal Cells at Physiological Concentrations

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ABSTRACT The antioxidant activity of flavonoids in cell-free systems has been studied extensively. We compared flavonoids with different structural features on their abilities to protect live Caco-2 intestinal cells from lipid peroxidation due to hydrogen peroxide and Fe²⁺ treatment. Flavonoids with o-dihydroxyl or vicinal-trihydroxyl groups, including quercetin, myricetin (flavonol), luteolin (flavone) and (−)-epigallocatechin gallate (EGCG; flavanol), when co-incubated with a mixture of 30 μmol/L H₂O₂ and 30 μmol/L FeSO₄, prevented the formation of malondialdehyde (MDA) at 1 or 10 μmol/L in at least one of two separate experiments. In experiments in which flavonoids were preincubated with cells but removed before the 30 μmol/L H₂O₂ and Fe²⁺ treatment, quercetin at 0.1 μmol/L, EGCG at 1 μmol/L and luteolin at 10 μmol/L exerted protective effects in at least one of two experiments. Kaempferol (flavonol) and the isoflavones, genistein and daidzein, did not prevent lipid peroxidation at 0.1–10 μmol/L in either co- or preincubation experiments. None of the flavonoids tested at 0.1–10 μmol/L increased H₂O₂ and Fe²⁺-induced lipid peroxidation after co- or preincubation. In summary, these observations support the importance of plant-based food items such as vegetables, fruits and teas in the diet. J. Nutr. 133: 2184–2187, 2003.

KEY WORDS: flavonoids • reactive oxygen species • iron • malondialdehyde • Caco-2 cells

Oxidative damage to various cellular components has been linked with the development of degenerative diseases (1). For example, unsaturated fatty acids in cell membranes can be oxidized in the presence of reactive oxygen species. The oxidation leads to the breakage of fatty acid chains and compromises the membrane integrity. In addition, the end product of lipid peroxidation, malondialdehyde (MDA), is cytotoxic (2). Greater intake of vegetables and fruits has been linked to a reduced incidence of diseases including cancer and cardiovascular diseases (3,4). The antioxidants in fruits and vegetables may contribute to the beneficial effects.

We investigated the ability of dietary flavonoids, a group of compounds found exclusively in plants, to prevent lipid peroxidation in live cells. Previous cell-free studies have demonstrated the ability of flavonoids to trap free radicals (5,6). In addition, flavonoids can prevent lipid peroxidation in microsomes and liposomes (5,7–9). The antioxidant potency of flavonoids in these studies depends on the arrangement of hydroxyl groups on the benzene ring (5–9). The first goal of this study was to examine whether in live cells that were incubated with reactive oxygen species, lipid antioxidant activity of flavonoids also followed the same structure-dependency as in the cell-free systems. Responses of live cells are complicated by the membrane permeability (10–12) and the intracellular metabolism of flavonoids (11,12). Nevertheless, experiments using live cells can provide more insight into the physiological importance of flavonoids. We used human intestinal Caco-2 cells as the model here because intestinal cells have the most exposure to dietary flavonoids under physiological conditions. Also, the transport, metabolism and biological activities of flavonoids have been characterized in Caco-2 cells (10–17).

An additional goal of this study was to determine whether earlier flavonoid exposure helps to protect the cells from lipid peroxidation. The concentration of flavonoids fluctuates in the intestinal lumen and plasma depending on the pattern of ingestion. The nutritional importance of flavonoids will be greater if intracellular flavonoids can protect cells even after the extracellular flavonoids have been removed. Cell membranes are permeable to flavonoids (10–12). In addition, cellular retention of quercetin was demonstrated in our previous fluorescence imaging and gene expression experiments (13,14,17). A recent study on oxidative DNA damage reported an antioxidant effect of (−)-epigallocatechin gallate (EGCG) after extracellular EGCG was removed. EGCG was the only flavonoid examined and the mechanism of the protection was unknown (18). We hypothesized that the lipid protective effect will occur after flavonoids in the medium have been removed.

The plasma concentrations of flavonoids are usually <10 μmol/L even after a bolus dose, and the steady-state levels are mainly <1 μmol/L (19–22). The intestinal luminal concentrations of flavonoids, although higher, probably do not exceed 10 μmol/L from a normal diet (<1 mg flavonoid), an amount suggested by extrapolation from the bolus dose study (21). Our study thus focused on the physiologically achievable concentration range of 0.1–10 μmol/L. Because information on the physiological actions of flavonoids is limited, we also investigated the plasma conditions (21).

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3 Abbreviations used: EGCG, (−)-epigallocatechin gallate; H₂O₂ + Fe²⁺, solution containing H₂O₂ and FeSO₄; MDA, malondialdehyde.
physiological concentrations of flavonoids has only recently become available (19–22), most earlier studies on flavonoids and lipid peroxidation had not considered physiological concentrations.

MATERIALS AND METHODS

Materials. Quercetin, myricetin, kaempferol and luteolin (90–98% purity) were purchased from Sigma-Aldrich (St. Louis, MO). Genistein, daidzein and EGCG (purity > 95%) were purchased from Calbiochem (La Jolla, CA). Hydrogen peroxide and ferrous sulfate (purity > 99%) were obtained from Fisher Scientific (Pittsburgh, PA). 1-Methyl-2-phenylimidazole and malondialdehyde bis(dimethyl acetal) (99% pure) used for MDA analysis were from Sigma-Aldrich. All cell culture reagents with the exception of fetal bovine serum were from Invitrogen (Carlsbad, CA). Fetal bovine serum was from Hyclone (Logan, UT). Distilled deionized water or 100% ethanol was used for solution preparation. All other chemicals used in the study were of reagent grade with >99% purity.

Cell culture and treatment. Human colon adenocarcinoma–derived Caco-2 cells were purchased from the American Type Culture Collection (Manassas, VA). These cells were cultured as described previously (13,15). Cells were seeded at 4 × 10^4 cells/cm^2 in 6-well plates and used for the experiment on day 8 after seeding. Under these seeding conditions, Caco-2 cells were confluent on day 4 and started to express maltase, the differentiation marker of enterocytes after reaching confluence (15). All flavonoids were prepared as 10 or 25 mmol/L sterile ethanol stock solutions and kept at −20°C (15,16). In the co-incubation experiment, flavonoids were diluted to the desirable concentrations in HBSS immediately before the experiment. They were added to the wells containing attached cells incubated in HBSS. Solutions containing H_2O_2 and FeSO_4 (H_2O_2/Fe^{2+}) were mixed in HBSS and added to the above wells 30 min later to initiate the oxidation reaction. The final concentrations of H_2O_2 and FeSO_4 were both 30 μmol/L. The concentrations of flavonoids used are indicated in Table 1. The cells were incubated for an additional 3 h before being lysed for the MDA assay. Hydrogen peroxide and ferrous sulfate solutions were freshly prepared for each experiment. All incubations were carried out inside the 37°C, 5% CO_2 cell culture incubator. Each treatment in an experiment was repeated three times with three independent wells of cells. Results from two independent experiments are shown.

TABLE 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Flavonol Quercetin</th>
<th>Flavanol EGCG</th>
<th>Flavone Luteolin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Malondialdehyde, pmol/mg protein</td>
<td>Malondialdehyde, pmol/mg protein</td>
<td>Malondialdehyde, pmol/mg protein</td>
</tr>
<tr>
<td>Co-incubation with flavonoids with H_2O_2 and FeSO_4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavanoid, μmol/L</td>
<td>0</td>
<td>654 ± 54</td>
<td>638 ± 114</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>675 ± 55</td>
<td>632 ± 65</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>548 ± 97</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>529 ± 85</td>
<td>641 ± 65</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>359 ± 88*</td>
<td>601 ± 65</td>
</tr>
</tbody>
</table>

| Preincubation with flavonoids before H_2O_2 and FeSO_4 | | | |
| Flavanoid, μmol/L | 0 | 654 ± 54 | 638 ± 114 | 527 ± 33 | 580 ± 81 | 580 ± 104 | 623 ± 221 |
| | 0.1 | 455 ± 76 | 496 ± 45 | 497 ± 59 | 544 ± 60 | 440 ± 83 | 615 ± 109 |
| | 1 | 710 ± 44 | 594 ± 113 | 279 ± 47* | 399 ± 11* | 501 ± 68 | 457 ± 61 |
| | 10 | 611 ± 97 | 641 ± 50 | 418 ± 97 | 614 ± 4 | 448 ± 20 | 329 ± 41 |

1 Values are means ± sd of triplicate wells of cells, n = 3. * Different from the respective control group without flavonoid (0 μmol/L), P < 0.05.
2 EGCG, (–)-epigallocatechin gallate; ND, not determined.

In the preincubation experiment, flavonoids at the concentrations indicated were included in the normal growth medium for 24 h. Cells were kept in the 37°C, 5% CO_2 incubator during this flavonoid pretreatment period. Media were then removed and cell monolayers were further rinsed with twice 37°C HBSS. After fresh HBSS was added to the wells with cells, freshly prepared H_2O_2 and FeSO_4 solutions were added at a final concentration of 30 μmol/L each. Cells were incubated for another 3 h inside the 37°C, 5% CO_2 cell culture incubator before being lysed for the MDA assay. Each treatment in an experiment was repeated three times with three independent wells of cells. Results from two independent experiments are shown.

Analytical methods. The extent of lipid peroxidation was determined by measuring the cellular content of MDA. We used a colorimetric assay (23) that is also available commercially as a kit for the determination of lipid peroxidation (#437634, Calbiochem). We followed the procedure of the kit with some modifications. Briefly, cells were rinsed twice with HBSS after the H_2O_2 + Fe^{2+} treatment. They were lysed in 20 mmol/L Tris buffer (pH 7.4) containing 5 mmol/L BHT through repeated freeze–thawing and a brief sonication. The cell lysate was then allowed to react with N-methyl-2-phenylimidazole for 30 min at 65°C. MDA content in the cell lysate was determined on the basis of the OD$_{586}$ of samples and the MDA standard curve (Fig. 1A). The linear regression of all MDA standard curves was 0.99. MDA in every cell lysate sample was determined by duplicate measurements and the mean of the duplicate measurements represented the result of an independent sample. Results from three independent samples were used to compute the mean and sd of the triplicate samples. A standard MDA curve was determined in every experiment. MDA production in the absence of H_2O_2 + Fe^{2+} and flavonoids (baseline MDA) was also measured in every experiment. Baseline MDA was subtracted from the total MDA to obtain values of H_2O_2 + Fe^{2+}–induced MDA production. The level of MDA was further normalized by the protein content of each sample as determined by the Lowry assay.

Statistical analysis. All results shown in the figures are means ± sd of three different wells of cells after subtracting the cellular basal MDA production measured in the absence of H_2O_2 + Fe^{2+} and flavonoid treatment. Statistical analyses were performed separately for two independent sets of data. One-way ANOVA was used followed by post-hoc Bonferroni–Dunn tests for comparisons with the respec-
We also tested the isoflavonoid treatment-induced lipid oxidation even after flavonoids were removed. These are the same flavonoids that showed antioxidant activity in the short-term co-incubation experiments (Table 1). It is thus possible that the mechanism mediating the antioxidant activities in these two types of experiments is the same. EGCG prevented chromosomal damage in live cells in both co-incubation and pre-incubation experiments (18). Here, EGCG prevented lipid peroxidation in live cells in both types of experiments (Table 1). Future studies are required to determine whether quercetin can also prevent DNA damage under both conditions. Quercetin metabolites were shown to have antioxidant activities in cell-free experiments (31,32). They may also have contributed to the antioxidant activity of quercetin in our study because intestinal cells can metabolize quercetin (11).

Flavonoids have oxidation potential and thus can be oxidized upon exposure to oxygen and reactive oxygen species. Flavonoids also have the iron-chelating property noted earlier (17,27–29), these experiments were carried out with higher concentration ratios of flavonoids to iron. In the current study, the iron concentration was 3- to 300-fold that of the flavonoids. It is thus unlikely that iron chelation contributed to the antioxidant activity observed here, especially in the experiments in which the medium flavonoids were removed before the addition of iron. It has been proposed, on the basis of an in vitro study, that flavonoids interact with the polar zone of phospholipid (30), and thus lipid solubility is critical for the antioxidant activity. Our observation is consistent with this model. Among the flavonoids tested here, myricetin had the lowest lipid solubility and daidzein the highest (26), yet lipid antioxidant activity was detected for both compounds. Quercetin, myricetin, luteolin and EGCG meet this criterion, whereas this feature is absent in kaempferol, genistein and daidzein. Some previous studies using cell-free systems or oxidation potential estimation have also predicted kaempferol and isoflavones to be weaker antioxidants (6,26).

Our study is the first to examine the structure-function relationship of flavonoids as lipid antioxidants in live cells. We chose to study this panel of flavonoids because they represented four different subclasses of flavonoids and they are present in the human diet. Also, these flavonoids were studied previously by other groups using cell-free systems (liposome or microsomal preparations), they all appeared to have lipid antioxidant activity (6–9). In our live cell experiment, we found that quercetin, myricetin, luteolin and EGCG inhibited lipid peroxidation in cells (Table 1) whereas kaempferol, genistein and daidzein did not at concentrations < 10 μmol/L (data not shown). When we analyzed the structural features of the 7 flavonoids tested in our study, we concluded that the most important criterion in protecting live cells from lipid peroxidation is the adjacent hydroxyl groups (o-dihydroxyl or vicinal trihydroxyl) in the ring structure. Quercetin, myricetin, luteolin and EGCG meet this criterion, whereas this feature is absent in kaempferol, genistein and daidzein. Some previous studies using cell-free systems or oxidation potential estimation have also predicted kaempferol and isoflavones to be weaker antioxidants (6,26).

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dizing or reducing agents under different conditions (26). In cell-free studies, quercetin and myricetin were shown to have lipid antioxidant activities at lower concentration (<10 μmol/L), yet they increased hydroxyl radical production and oxidative damage of DNA at 100 μmol/L (33). EGCG increased H₂O₂ production at concentrations >30 μmol/L, although it protected DNA at more physiological concentrations of <10 μmol/L (18). In our live cell experiment with concentrations of flavonoids up to 10 μmol/L, no increase in the MDA level occurred even after prolonged incubation (Table 1). Our results are thus consistent with the study of DNA damage (18) that showed that flavonoids can act as antioxidants with no prooxidant activity.

In conclusion, we observed the lipid antioxidant activity of these flavonoids. Previous studies were carried out mainly in cell-free systems. We found that lipid antioxidant activities of flavonoids occur in live cells with intact antioxidant systems in the range of physiologically achievable concentrations. Exposure to certain flavonoids can reduce H₂O₂ and Fe²⁺-induced lipid peroxidation even after the extracellular flavonoids have been removed. The observations here complement earlier EGCG study and extend our knowledge of the prevention of oxidative damage (18). These observations further support the importance of plant-based food items such as vegetables, fruits and teas in the diet.

LITERATURE CITED


