Bioactive Dietary Polyphenolic Compounds 
Reduce Nonheme Iron Transport across Human 
Intestinal Cell Monolayers

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Abstract
There is persuasive epidemiological evidence that regular intake of dietary bioactive polyphenolic compounds promotes human health. Because dietary polyphenolic compounds have a wide range of effects in vivo and vitro, including chelation of metals such as iron, it is prudent to test whether the regular consumption of bioactive polyphenolic components impair the utilization of dietary iron. We examined the influence of the dietary polyphenols (-)-epigallocatechin-3-gallate (EGCG) and grape seed extract (GSE) on transepithelial iron transport in Caco-2 intestinal cells. The range of EGCG and GSE concentrations used in this study was within physiological levels and did not affect the integrity of differentiated Caco-2 cell monolayers. Both EGCG and GSE decreased (P<0.001) transepithelial iron transport. However, apical iron uptake was increased (P<0.001) by the addition of EGCG and GSE. The increased uptake of iron might be due in part to the reducing activity of EGCG and GSE. Both EGCG and GSE reduced ~15% of the applied Fe3+ to Fe2+ in the uptake buffer. Despite the increased cellular levels of 55Fe, the transfer of iron across the basolateral membrane of the enterocyte was extremely low, indicating that basolateral exit via ferroportin-1 was impaired, possibly through formation of a nontransportable polyphenol-iron complex. Our data show that polyphenols inhibit nonheme iron absorption by reducing basolateral iron exit rather than by decreasing apical iron import in intestinal cells. J. Nutr. 138: 1647–1651, 2008.

Introduction
Bioactive dietary polyphenols are receiving increasing interest from scientists and consumers due to their reported health benefits for a variety of disorders (1–4). They are naturally occurring chemicals found in foods such as fruits, some types of grain, wine, and tea. Tea, made from the leaves of the plant Camellia sinensis, is a popular beverage worldwide. The beneficial effects of green tea are attributed to its polyphenolic compounds, particularly the catechins. (-)-Epigallocatechin-3-gallate (EGCG), the most abundant green tea catechin, is regarded as the most bioactive disease-preventing polyphenol compound in green tea. Grape seed extract (GSE), which contains various polyphenols including gallic acid, catechin, EGCG, EGC, epicatechin-3-gallate, epicatechin, and proanthocyanidins, also has been reported to have protective effects on various forms of cardiac disorders (5–7). Polyphenol-rich extracts derived from both green tea and grape seed have the potential to reduce oxidation of foods and beverages and are widely used as supplements or food additives. Their antioxidant activities have been shown to be dependent on the ability of their constituent phenolic compounds to scavenge free radicals and to chelate metals such as iron (8,9).

Because of the increasing interest in EGCG and GSE as dietary supplements and food additives and a growing understanding of the potential health benefits, we determined the potential effects of the bioactive dietary polyphenols EGCG and GSE on iron absorption by utilizing the human intestinal Caco-2 cell line as a model system to study this process. On confluence, these cells spontaneously differentiate to exhibit many of the morphological and functional features of normal mature small intestinal enterocytes and have been widely used as a model of normal human intestinal epithelium (10,11). In particular, fully differentiated Caco-2 cells are a well-established in vitro model of human intestinal iron absorption (12–14). As in the human intestine, Caco-2 cells display enhanced transepithelial transport of iron in iron-depleted cells (15,16) and express divalent metal transporter-1 (DMT-1), duodenal ferrireductase, ferroportin-1 (FPN-1), hephaestin, transferrin receptor-1, and ferritin (12,17,18), which are involved in iron absorption and metabolism.
It is thought that dietary iron in the lumen is reduced to ferrous ion by either ferrireductase activity on the cell surface (13,19,20) or exogenous dietary reducing agents, such as ascorbic acid (19). Iron is then transported across the apical membrane via the apical iron transporter, DMT-1, into the enterocyte (21,22). The newly transported iron is then distributed to the basolateral surface or to iron-binding proteins (e.g., heme, nonheme iron-binding proteins, and ferritin). Finally, the newly acquired iron is transferred across the basolateral membrane of the enterocyte via the iron exporter FPN-1 (23–25) and then oxidized to ferric ion by the hephaestin (26) on the basolateral surface prior to release into the circulation.

Iron is an essential trace element for human life. Although iron is quite abundant in the environment, iron deficiency is still the most common nutritional deficiency world-wide due not only to low intake of this essential trace metal but often to poor bioavailability as well. Because the antioxidant properties of the polyphenols include chelation of metals such as iron, it is prudent to examine the effects of the bioactive phenolic compounds on intestinal iron absorption. The objective of this study was to examine the effects of ECGG, the major bioactive polyphenolic compound of green tea, and GSE on the absorption of iron utilizing intestinal Caco-2 cells.

Materials and Methods

Reagents. Tissue culture media and Hanks’ balanced salts solution (HBSS), glutamine, nonessential amino acids, and penicillin/streptomycin were purchased from Invitrogen. Fetal bovine serum was obtained from Hyclone. ECGG (TEAVIGO, >95% pure) and GSE were obtained from DSM Nutritional Products and Partoeno, respectively. The chemical characteristics and degree of polymerization for the GSE used in these studies has been documented (27). The FeSO₄ (as FeCl₃) was purchased from PerkinElmer. Unless otherwise noted, all other reagents were purchased from Sigma Chemical, Fisher Scientific, or VWR.

Cell culture. The human Caco-2 cell line was purchased from American Type Culture Collection. Stock cultures were maintained at 37°C in complete medium in a humidified atmosphere of 95% air and 5% CO₂ and employed for experiments within 20 serial passages. The complete culture medium contained DMEM supplemented with 25 mM/L glucose, 2 mM/L glutamine, 100 mM/L nonessential amino acids, 100 U/L penicillin G, 100 mg/L streptomycin, and 10% fetal bovine serum. Stock cultures were seeded at 10,000 cells/cm² and at ~85% confluence they were split by treatment with 0.5 g/L trypsin-0.5 mmol/L EDTA in HBSS. m Cells are fully differentiated at 17 postconfluence for experiments. The Caco-2 cell monolayer formed tight junctions at d 17 postconfluence as defined by the transepithelial electrical resistance (TEER) values of >250 Ω/cm². Cells are fully differentiated at d 17 after confluence in normal cell culture conditions (12,28).

55Fe transport and uptake. Transepithelial iron transfer from the apical compartment to the basolateral compartment and cellular iron accumulation were determined (12,19,29). After washing the cell monolayer 3 times with Ca²⁺- and Mg²⁺-free HBSS at 37°C, cells were incubated at 37°C with 1.5 mL of 10 μmol/L Fe²⁺ nitrilotriacetic acid (NTA) in iron-uptake buffer containing the indicated bioactive compounds in the apical compartment and 2.5 mL DMEM in the basolateral compartment. All test solutions were freshly prepared before each experiment. Stock solutions of 10 mM/L FeCl₃·6H₂O and 20 mM/L NTA were diluted 10-fold in sterile, deionized water. The diluted stock solutions were mixed with 55FeCl₃ (specific radioactivity, 192 Bq/mmol; PerkinElmer) to provide 37 kBq per well for uptake and transport studies. The uptake buffer contains 130 mM/L NaCl, 10 mM/L KCl, 1 mM/L MgSO₄, 5 mM/L glucose, and 50 mM/L HEPES, pH 7.0. A quantity of 200 μL was removed from the basolateral chamber every hour and replaced with an equivalent volume of prewarmed DMEM; time course data were corrected to account for this sample replacement. The rate of radiolabeled iron transfer across the cell monolayer was increased during the 3-h incubation and transport rates [μmol/hr · mg cellular protein] were calculated by linear regression analysis (r² > 0.995). The integrity of tight junctions between cells was monitored by measuring TEER and phenol red transport; any leaking cell monolayers were discarded. To measure the cellular levels of 55Fe, we washed cell monolayers 3 times with ice-cold wash buffer containing 150 mM/L NaCl, 10 mM/L HEPES, pH 7.0, and 1 mM/L EDTA to remove any nonspecifically bound radioisotope. This washing step was effective in removing all surface-bound iron, because additional wash steps using solution containing 100 μmol/L bathophenanthroline disulfonate (Fe²⁺ chelator) or desferrioxamine (DFO; Fe³⁺ chelator) did not further change cellular 55Fe content after the wash. Cells were homogenized in PBS containing 1 mM/L EDTA and 0.2% Triton X-100 and 55Fe was quantified by liquid scintillation counting in glass vials. Cellular protein levels were assessed using Bio-Rad protein assay kit (Bio-Rad Laboratory). The levels of EGCG (46 mg/L = 100 μmol/L) and GSE (46 mg/L) used for iron uptake and transport studies are within physiological levels, because 1 cup (200 mL) of green tea contains up to 100–200 mg EGCG and most GSE supplements contain 100–500 mg GSE per capsule.

Assay for Fe³⁺ reduction. To assess Fe³⁺ reduction activity, ECGG and GSE at various concentrations (0–46 μg/L) were added to test tubes containing 10 μmol/L Fe³⁺ (NTA) and 100 μmol/L ferrozine (FZ) and incubated for 1 h at 37°C. After incubation, the concentration of Fe²⁺ was immediately determined by monitoring the formation of Fe²⁺-FZ complex at 562 nm. Absorbance (562 nm) readings were compared with known concentrations of Fe²⁺-FZ complex. Standards were prepared by adding 100 μmol/L ascorbic acid to uptake buffer containing both Fe³⁺ (NTA) at the various concentrations (0–10 μmol/L) and 100 μmol/L FZ, followed by incubation for 20 min at room temperature.

Fe²⁺-chelating activity. The chelation of ferrous ion by ECGG or GSE was estimated as previously described (30,31). Briefly, the bioactive compounds at the indicated concentrations were mixed with FZ (100 μmol/L) in the uptake buffer (pH 7) followed by the addition of 10 μmol/L FeSO₄ as a source of ferrous ion. After incubation for 20 min, the formation of the Fe²⁺-FZ complex was spectrophotometrically determined by reading the solution at 562 nm. The percentage of Fe²⁺-chelating effect was calculated as follows: chelating effect (%) = [1 – (absorbance of sample with bioactive component at 562 nm)/absorbance of control without bioactive compound at 562 nm)] × 100. The absorbance (562 nm) readings for the Fe²⁺-FZ complex were linear within the concentrations used in this study.

Statistical analysis. Values were expressed as means ± SEM, n = 4–6. Data were analyzed using 1- or 2-way (treatment × time) ANOVA with the following Bonferroni’s multiple comparison tests post hoc for multiple comparisons using Prism 5.0 software (GraphPad). Data were log-transformed as necessary to attain homogeneity of variance and data are reported as nontransformed means. The REG (regression) procedure was used to perform simple linear regression analysis (Table 1). Differences were considered significant at P < 0.05.

Results

EGCG and GSE inhibit transepithelial iron transport. The quantity of 55Fe transferred from the apical to the basolateral compartment of the Caco-2 cell monolayer increased between 1 and 3 h of incubation. The addition of either polyphenol to the uptake buffer decreased (P < 0.001) the rate of 55Fe transfer across the cell monolayer. Interestingly, the rate of iron transfer was lower (P < 0.05) in the presence of ECGG than in the presence of GSE. During 3 h of incubation, both ECGG and GSE...
TABLE 1  The inhibitory effects of EGCG and GSE on the rate of apical 56Fe transfer across fully differentiated Caco-2 cell monolayers.1,2

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Rate of 56Fe transfer (AP to BL chamber) pmol/(h · mg cellular protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.08 ± 0.65a</td>
</tr>
<tr>
<td>+ EGCG (46 mg/L)</td>
<td>0.31 ± 0.08b</td>
</tr>
<tr>
<td>+ GSE (46 mg/L)</td>
<td>1.18 ± 0.09b</td>
</tr>
</tbody>
</table>

1 Data are means ± SEM, n = 6. Means without a common letter differ, P < 0.05.
2 The rate of transepithelial 56Fe transport across the differentiated Caco-2 cell monolayers was calculated during 3-h incubation by linear regression analysis (control, r² = 0.995; + GSE, r² > 0.999; + EGCG, r² > 0.999). AP: apical compartment; BL: basolateral compartment.

Discussion

Our unexpected finding in this study was that the iron transferred across the basolateral membrane of the enterocyte-like Caco-2 cells was extremely low despite the polyphenol-assisted uptake from the apical side. Most dietary factors that inhibit iron absorption have to date been characterized by their ability to prevent apical iron uptake, typically by chelating iron in the gastrointestinal lumen and limiting access to the apical surface of enterocytes (i.e. phytic acid and tannic acid), whereas other dietary factors such as ascorbic acid and some amino acids enhance iron absorption by stimulating apical uptake iron (32–34). It is possible that polyphenols form complexes with iron in the cell and prevent iron exit across the basolateral membrane via FPN-1 in Caco-2 cells. Because the potential chelating activity of EGCG and GSE for iron was detected in this investigation, it is likely that such activity does contribute to the blockage of iron from exit across the basolateral membrane. The levels of EGCG and GSE used in this study were within physiological levels and did not affect the integrity of Caco-2 cell monolayers. The levels of FPN-1, ferritin, and transferrin receptor-1 protein were not changed by EGCG or GSE during the iron transport study (E-Y. Kim and O. Han, unpublished data). A previous study (33) of decreased transepithelial iron transfer across the cell monolayer by 94 ± 0.1 and 90 ± 0.1%, respectively (Fig. 1). The addition of bioactive dietary polyphenols did not alter TEER values, thereby confirming the integrity of the monolayer for EGCG or GSE-added cells.

Apical iron uptake is increased by EGCG and GSE. The addition of EGCG and GSE enhanced (P < 0.001) the uptake of iron across the brush border membrane and its assimilation by Caco-2 cells to 6.3- and 16.2-fold of control, respectively, during the 3-h assay. The control cells accumulated 35 ± 4.5 pmol/mg cellular protein in 3-h incubation time. These are unexpected results, because most dietary factors alter iron absorption by modulating uptake of iron across the apical membrane of the enterocyte. To examine potential mechanisms by which these polyphenolic compounds increase iron uptake, we next tested their biochemical properties.

Fe²⁺-reducing activity of EGCG and GSE. The formation of Fe²⁺ was estimated by adding FZ to iron uptake solution containing different concentrations of EGCG or GSE. The levels of Fe²⁺-FZ were augmented by increasing concentrations of EGCG or GSE in the test solution (Fig. 2). The addition of 46 mg/L of EGCG and GSE reduced a similar amount (P > 0.05) of Fe²⁺ to Fe²⁺ in iron uptake buffer containing 10 μmol/L Fe³⁺(NTA)₂. Both EGCG and GSE increased the formation of Fe³⁺-FZ in a dose-dependent manner.

Fe²⁺-chelating activity of EGCG and GSE. Because the transport of ⁵⁹Fe across the cell monolayer was significantly decreased by EGCG and GSE, we assessed the chelating activity of these bioactive compounds by evaluating their ability to compete with FZ for Fe²⁺ ion (30,31). The chelating potency of EGCG was much higher (P < 0.05) than that of GSE (Fig. 3). The addition of EGCG chelated ~1-fold more iron than GSE from iron uptake buffer containing 10 μmol/L FeSO₄. A positive control experiment with same concentration of DFO showed that a chelating potency of EGCG is similar to that of DFO (data not shown).

![FIGURE 1](image1.png) The inhibitory effects of EGCG and GSE on iron transport across fully differentiated Caco-2 cell monolayers. Values are means ± SEM, n = 4. Means at a time without a common letter differ, P < 0.05. Within a treatment, means without a common symbol differ, P < 0.05.

![FIGURE 2](image2.png) Fe²⁺-reducing activity of various concentrations of EGCG (A) and GSE (B). Incubations included 10 μmol/L Fe³⁺(NTA)₂ and 100 μmol/L FZ. Data are means ± SEM, n = 4. Means without a common letter differ, P < 0.05.
mice on bioavailability of dietary polyphenols reported that although bioavailability of polyphenols was very low, a vast amount of polyphenols accumulated in the intestinal tissue, which supports the possibility that polyphenols form complexes with iron and prevent iron exit via FPN-1 in Caco-2 cells. Future studies on mechanisms of polyphenol metabolism and absorption should further provide insight into the mechanisms by which polyphenols modulate dietary iron absorption and metabolism. Our results show that polyphenols inhibit nonheme iron absorption by reducing basolateral iron exit rather than by decreasing apical iron import in intestinal cells.

An interesting observation in this study was that the cellular iron uptake was strikingly increased in Caco-2 cells by EGCG and GSE. This factor was initially identified based on increase of iron uptake by dietary factors containing reducing properties. Ascorbic acid stimulates apical iron uptake by reducing dietary ferric ion to ferrous ion. The effects of EGCG and GSE on apical iron uptake were comparable to that of ascorbic acid (unpublished data, E-Y. Kim and O. Han). One molecule of EGCG results in the reduction of 4 Fe$^{3+}$ ions (36). However, under our experimental conditions, the reducing capacity of EGCG and GSE was much lower than that of ascorbic acid. Whereas 100 μmol/L of ascorbic acid reduced 100%, the same concentration of EGCG and GSE reduced only 14.3 and 15.4% of Fe$^{3+}$, respectively, when 10 μmol/L Fe$^{3+}$ (NTA)$_2$ was applied. One possibility is that reduced ferrous ion is associated with polyphenols and then enters into the cell. Our unpublished data suggest that perhaps polyphenols increase apical iron uptake via non-active transport pathway. For example, bioactive polyphenolic compounds also increased the apical iron uptake at the room temperature. A previous study (31) of brain cell iron chelation suggested that polyphenols can be membrane permeable and therefore it is possible that EGCG and GSE may enter cells as complex forms with Fe$^{2+}$. Polyphenols including EGCG have been shown to chelate metals such as iron (8,37–41). It is possible that the greater decrease of the basolateral $^{55}$Fe transport by EGCG may be due to the greater iron-chelating potency of EGCG compared with GSE. Further research on the uptake of iron as Fe$^{2+}$-polyphenols and Fe$^{3+}$-polyphenol complexes, as well as DMT-1-mediated free Fe$^{2+}$ uptake, should provide further insight into the process of dietary iron uptake in the presence of bioactive dietary polyphenols.

Because dietary polyphenolic compounds have a wide range of effects in vivo and vitro, including metal-chelating activities, a high intake of dietary polyphenolic compounds may have important consequences on iron status. For example, tea, red wine, and other beverages rich in phenolic compounds, including coffee, are known to inhibit the absorption of nonheme iron (42–46). A significant decrease of iron absorption was observed in humans when the test meal was accompanied by black tea instead of water (42). An extract of green tea also significantly decreased intestinal iron absorption in humans (43,47) as well as in animals (44). Coffee also was shown to inhibit iron absorption in humans in a dose-dependent manner (45). Similar to tea and coffee, red wine also inhibits iron absorption (46). It was suggested that the content and type of polyphenols present in foods will determine this inhibitory effect. However, the precise mechanism by which bioactive dietary polyphenolic compounds inhibit iron absorption has not been delineated. This is the first report to our knowledge demonstrating that bioactive polyphenols inhibit iron transport across the enterocyte by decreasing basolateral iron exit.

**Literature Cited**


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