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Bioactive Dietary Polyphenols Inhibit Heme Iron Absorption in A Dose-Dependent Manner in Human Intestinal Caco-2 cells

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Abstract

Although heme iron is an important form of dietary iron, its intestinal absorption mechanism remains elusive. Our previous work revealed that (–)-epigallocatechin-3-gallate (EGCG) and grape seed extract (GSE) markedly inhibited intestinal heme iron absorption by reducing the basolateral iron export in Caco-2 cells. The aims of this study were to examine whether small amounts of EGCG, GSE and green tea extract (GT) could inhibit heme iron absorption, and to test whether the inhibitory action of polyphenols could be offset by ascorbic acid. A heme-⁵⁵Fe absorption study was conducted by adding various concentrations of EGCG, GSE and GT to Caco-2 cells in the absence and presence of ascorbic acid. Polyphenolic compounds significantly inhibited heme-⁵⁵Fe absorption in a dose-dependent manner. The addition of ascorbic acid did not modulate the inhibitory effect of dietary polyphenols on heme iron absorption when the cells were treated with polyphenols at a concentration of 46 mg/L. However, ascorbic acid was able to offset or reverse the inhibitory effects of polyphenolic compounds when lower concentrations of polyphenols were added (≤ 4.6 mg/L). Ascorbic acid modulated the heme iron absorption without changing the apical heme uptake, the expression of the proteins involved in heme metabolism and basolateral iron transport, and heme oxygenase activity, indicating that ascorbic acid may enhance heme iron absorption by modulating the intracellular distribution of ⁵⁵Fe. These results imply that the regular consumption of dietary ascorbic acid can easily counteract the inhibitory effects of low concentrations of dietary polyphenols on heme iron absorption but cannot counteract the inhibitory actions of high concentrations of polyphenols.

Keywords

heme iron absorption; ascorbic acid; green tea; (–)-epigallocatechin-3-gallate (EGCG); grape seed extract

Introduction

The regulation of systemic iron homeostasis is crucial, as iron deficiency leads to anemia and as iron toxicity results in hemochromatosis (Britton et al., 1994). In both eukaryotic cells and most prokaryotic cells, iron is notably required for survival and proliferation, as a member of a diverse group of hemoproteins that includes proteins involved in oxygen transport and storage (hemoglobin and myoglobin), electron transfer (cytochromes) and

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Practical Application:

Bioactive dietary polyphenols inhibit heme iron absorption in a dose-dependent manner. The small amounts of polyphenolic compounds present in foods are capable of reducing heme iron transport across the intestinal enterocyte. However, the inhibitory effects of dietary polyphenolic compounds on heme iron absorption can be offset by ascorbic acid and can possibly be avoided by decreasing the consumption of polyphenols while simultaneously taking ascorbic acid.

DNA synthesis (ribonucleotide reductase) (Hentze et al., 2004). Currently, iron deficiency is one of the most common diet-related health problems worldwide; in a severe stage, it leads to anemia, which is a major public health concern affecting up to 2 billion people (WHO, 2008). Systemic iron homeostasis requires mechanisms for regulating iron entry into and mobilization from stores to counteract iron loss (1–2 mg/day) and to compensate for the daily production of 200 billion new erythrocytes, along with other uses (Hentze et al., 2004). Because there is no efficient way to eliminate iron from the body, intestinal iron absorption is tightly modulated (and even self-modulated) to maintain iron homeostasis (Hentze et al., 2004). There are two types of dietary iron: heme iron and non-heme iron (Beard and Han, 2009). The importance of dietary heme iron cannot be underestimated. In the Western diets of average nonvegetarians, heme iron constitutes one-third of the total dietary iron but two-thirds of the total absorbed iron, indicating that heme iron is more efficiently absorbed than is non-heme iron (Bezwoda et al., 1983; Carpenter and Mahoney, 1992).

Heme is initially taken up from the intestinal lumen into enterocytes as an intact metalloporphyrin (Conrad et al., 1967). There are currently two prevailing hypotheses regarding the mechanism of heme uptake into enterocytes. The long-standing hypothesis is that uptake occurs via receptor-mediated endocytosis, by which heme is internalized into endosomes and then degraded by heme oxygenases (HOs) to release free ferrous iron, which is presumably transported to the cytoplasm by divalent metal transporter 1 (DMT1) (Weintraub et al., 1968). Alternatively, heme iron is proposed to be directly taken up by proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1) into the cytoplasm, where it is catabolized to biliverdin and free ferrous iron by HOs (Shayeghi et al., 2005). Despite differences in the potential mode of uptake, heme-derived free iron in the enterocytes ultimately joins the labile iron pool, which is ready to be incorporated into ferritin (Ft) for transient storage or to be exported across the basolateral membrane via ferroportin-1 (FPN-1) into circulation, where it is oxidized to ferric iron by hephaestin (HEPH) and bound by transferrin (Tf) (Vulpe et al., 1999; Donovan et al., 2000).

Bioactive dietary polyphenols have attracted increasing attention recently due to their reported health benefits (Frederiksen et al., 2007; Bose et al., 2008). Because they also possess the capability to chelate metals (Mandel et al., 2006), it would be interesting to investigate the relationship between dietary polyphenols and intestinal iron absorption. A high intake of dietary polyphenolic compounds may have important consequences on iron status. For example, tea, red wine and other beverages that are rich in polyphenolic compounds are known to inhibit the absorption of non-heme iron (Cook et al., 1995; Hurrell et al., 1999; Kim et al., 2008; Thankachan et al., 2008). However, there is limited information on the effect of bioactive polyphenols on heme iron absorption. Our previous study found that 46 mg/L both (–)-epigallocatechin-3-gallate (EGCG) and grape seed extract (GSE) almost completely blocked heme iron absorption by decreasing the basolateral iron export in Caco-2 cells (Ma et al., 2010). These findings suggested that the content and type of polyphenols present in foods will determine the inhibitory effect on iron absorption. However, the precise mechanism by which bioactive dietary polyphenolic compounds inhibit heme iron absorption has not been determined. The objectives of this study were to investigate whether the inhibitory effects of polyphenols can be offset by dietary ascorbic acid and whether lower concentrations of these polyphenolic compounds are still capable of inhibiting the heme iron absorption of Caco-2 cells. Because green tea is a major natural source of EGCG and is a popular beverage in a number of countries in which iron deficiency is a major nutritional problem, the effect of green tea extract (GT) on heme iron absorption and its relationship to dietary ascorbic acid were also examined.

Materials and Methods

Reagents

EGCG (TEAVIGO™, purity >95%), GSE and GT were purchased from DSM Nutritional Products (Parsippany, NJ), Partoeno (Bordeaux, France) and Pharmanex Inc. (Provo, UT), respectively. The chemical characteristics and the degree of polymerization for the GSE used in these studies have been documented (Tsang et al., 2005). GT is a mixture of catechins, including EGCG as the major component (43.0% by weight), followed by epicatechin-3-gallate (13.7%), epicatechin (6.0%), gallocatechin gallate (5.6%), epigallocatechin (4.0%), gallocatechin (2.3%), catechin (2.0%) and catechin gallate (1.4%) (Lu et al., 2005). In this study, the concentration of EGCG, GSE or GT was expressed as the weight in buffer bathing the apical side of the cells [mg/L]. The ^{55}Fe (in the form of $^{55}\text{FeCl}_3$) was obtained from PerkinElmer (Boston, MA). Hanks' balanced salts solution (HBSS), glutamine, nonessential amino acids and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Unless otherwise noted, all other reagents were obtained from Sigma Chemical (St Louis, MO), VWR (West Chester, PA) or Fisher Scientific (Springfield, NJ).

Caco-2 cell culture

The human Caco-2 (HTB-37™) cell line was purchased from American Type Culture Collection (Rockville, MD) and was maintained at 37°C in complete medium in a humidified atmosphere of 95% air and 5% CO₂. The complete culture medium consisted of Dulbecco's Modified Essential Medium (DMEM), supplemented with 25 mmol/L glucose, 2 mmol/L glutamine, 100 µmol/L nonessential amino acids, 100 U/L penicillin G, 100 mg/L streptomycin and 10% FBS. The stock cultures were seeded at 10,000 cells/cm² and were split at ~85% confluence by treatment of 0.5 g/L trypsin and 0.5 mmol/L EDTA in HBSS. For experiments, 50,000 cells/cm² in a volume of 1.5 mL complete medium were seeded on a 3 µm microporous membrane insert (4.9 cm², BD Biosciences, Bedford, MA) coated with collagen (5 µg/cm²) (BD Biosciences, Bedford, MA) on a 6-well plate. The basolateral chamber contained 2.5 mL complete DMEM. The culture medium was changed every other day, and cells were utilized for heme-iron transport experiments after 17 d post-confluence. The Caco-2 cells are fully differentiated at 17 d post-confluence in normal cell culture conditions (Louvard et al., 1992). The cell monolayer formed tight junctions at 17 d post-confluence, as defined by the transepithelial electrical resistance values of >250 Ω/cm².

MEL cell culture and hemoglobin (Hb) synthesis

The murine erythroleukemia (MEL) cell line was a generous gift from Dr. Robert Paulson at Pennsylvania State University. The MEL cells were grown in the same conditions as described above for the Caco-2 cells. As previously reported, Hb was synthesized using MEL cells (Ma et al., 2010). Briefly, to induce Hb synthesis, erythroid differentiation of cells was induced by adding dimethyl sulfoxide (DMSO) as previously reported (Ma et al., 2010). (^{55}Fe)₂-Tf was prepared from apo-Tf and ^{55}Fe and purified as described previously (Ma et al., 2010). The level of Tf saturation was estimated from the A₄₆₅/A₂₈₀ ratio, which was routinely found to be 0.046, consistent with complete saturation on both sites of Tf for iron binding (Huebers et al., 1978). To produce ^{55}Fe -Hb, MEL cells were seeded at 10,000 cells/cm² and were treated with 2 µmol/L (^{55}Fe)₂-Tf and 2% DMSO. After a 6-d treatment, the cells were harvested and washed three times with phosphate-buffered saline (PBS, pH 7.0) and were then collected by centrifugation for 5 min at 800 ×g, 4°C.

Spectrophotometric quantification of Hb and Hb digestion

Pellets of MEL cells ($\sim 70 \times 10^6$ of cells) harvested after the 6 d treatment were resuspended in 0.5 mL distilled water and were lysed by 4 cycles of freezing (in dry ice/ethanol for 3 min) and thawing (at 37°C for 3 min). The cell lysate was centrifuged at 20,000 xg for 15 min at 4°C using an Eppendorf 5417 centrifuge (Hamburg, Germany), and 300 μ L supernatant was transferred to a new tube. The benzidine assay was performed in duplicate by adding the reagents in the following order: 10 μ L supernatant, 900 μ L deionized water and 100 μ L freshly prepared 10 mg/ml benzidine with 0.5% acetic acid. The reaction was initiated by the addition of 40 μ L 30% H₂O₂. After exactly 90 sec, the absorbance was measured at 604 nm. The Hb concentration was then calculated from the measured absorbance using a calibration curve obtained with purified Hb standards measured in the same way as the supernatant. Hb-⁵⁵Fe solution, prepared from MEL cell lysate, was digested, and the digestion rate was estimated by measuring the remaining Hb content in the supernatant using a benzidine assay as described above. Pellets of heme were dissolved in 10 mmol/L of NaOH and were further diluted in iron uptake buffer (final pH 7.0). The ⁵⁵Fe-heme specific activity was between 0.4 Ci/mol and 0.45 Ci/mol heme.

Transepithelial heme-⁵⁵Fe transport

Transepithelial heme-derived ⁵⁵Fe transfer from the apical to the basolateral compartment was determined by scintillation. After washing the Caco-2 cell monolayer three times with Ca²⁺- and Mg²⁺-free HBSS, the cells were incubated at 37°C with 1.5 mL iron uptake buffer containing 1 μ mol/L ⁵⁵Fe-heme and the indicated bioactive compounds in the apical compartment and 2.5 mL DMEM in the basolateral compartment for 7 h. The iron uptake buffer (pH 7.0) consisted of 130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L MgSO₄, 5 mmol/L glucose and 50 mmol/L HEPES. During the 7 h incubation, 200 μ L media was transferred from the basolateral chamber to glass vials at the indicated time points (1, 3, 5 and 7 h) and was replaced by an equivalent volume of pre-warmed DMEM; the time course data were corrected to account for this sampling replacement. The ⁵⁵Fe transport across the cell monolayer was linearly increased during a 7 h incubation. To measure the cellular levels of ⁵⁵Fe, cell monolayers were washed three times with ice-cold wash buffer to remove any nonspecifically bound radioisotope. The wash buffer (pH 7.0) contained 150 mmol/L NaCl, 10 mmol/L HEPES and 1 mmol/L EDTA. This washing step effectively removed all of the surface bound iron because additional wash steps using solution containing 100 μ mol/L bathophenanthroline disulfonate (BPS, Fe²⁺ chelator) and desferrioxamine (DFO, Fe³⁺ chelator) were not able to further reduce the cellular ⁵⁵Fe content after the wash. The cells were homogenized in PBS containing 0.3% Triton X-100, and ⁵⁵Fe was quantified by liquid scintillation counting in glass vials. Cellular protein levels were consistently 1.2 ± 0.2 mg/well, as assessed by the Bio-Rad protein assay kit (BioRad Laboratory Inc., Hercules, CA). The level of heme iron (1 μ mol/L) used for iron transport studies is similar to that obtained from a meal containing 10 g of cooked beef (Hazell et al., 1982). The concentrations of polyphenolic compounds used in this study are within physiological ranges. A green tea bag contains up to 20–200 mg EGCG. The contents of extracted polyphenols in tea are dependent on the origin, manufacture, and brew conditions (such as the water temperature, brew time and tea to water ratio) of the tea (Astill et al., 2001). Thus, a cup of green tea can provide a range of EGCG content, from 1 to 200 mg EGCG. Most GSE supplements contain 100 to 500 mg GSE per capsule. However, red wine contains about 132–366 mg/L catechin equivalents, and white wine contains 5–13 mg/L catechin equivalents (Sanchez-Moreno et al., 2003). Because the total gastric volume of a meal can be 1–2 L, depending on the amount of food consumed (Burton et al., 2005), the concentrations of polyphenols (0.46–46 mg/L) used in the present study are within practical ranges. Similarly, because the recommended dietary allowance of ascorbic acid is 75–90 mg (426–511 μ mol) for adults,

the concentration of ascorbic acid (100 $\mu\text{mol/L}$) used in the current study is also within practical ranges.

Western blot

Western blot analysis was performed to determine the protein levels of HO-1, HO-2, FPN-1, Heph, transferrin receptor1 (TfR1) and Ft in Caco-2 cells, as previously described (Han and Kim, 2007; Ma et al., 2010). Protein samples were extracted from Caco-2 cells treated with heme and various polyphenols in the absence or presence of ascorbic acid for 7 h. The total protein concentrations were determined using a Bio-Rad protein assay kit. The protein samples (40 μg) were mixed with Laemmli buffer, boiled for 10 min, resolved by a 12% SDS-PAGE and transferred to nitrocellulose membranes by electroblotting. The membranes were stained with reversible Ponceau dye for 5 min to confirm an equal amount of total proteins for each lane and were washed several times by 10 mmol/L Tris-base and 150 mmol/L NaCl (TBS, pH 7.4) containing 0.05% Tween 20 (TBST). The membranes were first blocked by 5% nonfat powdered milk in TBS at room temperature for 1 h and were then incubated for 2 h at room temperature with an affinity-purified HO-1, HO-2, FPN-1, Heph, TfR1 or Ft antibody (1:2000) in TBST. The membranes were washed several times with TBST and were then incubated for 1 h at room temperature with peroxidase-linked secondary antibody (1:3000) in TBS containing 5% nonfat powdered milk. The antigens were visualized by enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA), detected using a ChemiDoc XRS system (Bio-Rad, Hercules, CA) and quantified using Quantity-One application software (Bio-Rad Hercules, CA). The membranes were stripped and reprobed with anti-calnexin antibody (Sigma, St. Louis, MO) to confirm equal loading and transfer.

HO activity assay

HO activity was assessed on the basis of the rate of bilirubin production (Maines, 1996). HO activity reflects the total activity of both HO-1 and HO-2 in the cell. Caco-2 cells treated with polyphenols in the presence or absence of ascorbic acid for 7 h were harvested in ice-cold 0.3 mL PBS with 1% Triton X-100 and homogenized. The cell lysates were incubated with 10 $\mu\text{mol/L}$ hemin at 37°C with shaking for 30 min. The reaction was terminated by placing the samples on ice. The samples were centrifuged at 4°C for 10 min to remove the cell debris. The amount of bilirubin in the supernatant was determined using the QuantiChrom™ bilirubin assay kit (BioAssay Systems, Hayward, CA). All of the assays were conducted under dim light. The HO activity was expressed as nmol bilirubin formed/min/mg protein.

Statistical analysis

Values were expressed as means $\pm$ SEM, with $n = 3-6$. The experiments were repeated at least three times. Data were analyzed by a 2-way ANOVA using R software and by a 1-way ANOVA with the following post-hoc tests for multiple comparisons using Prism 5.0 software (GraphPad). Differences were considered significant when $P < 0.05$.

Results

Effect of ascorbic acid on inhibitory action of EGCG and GSE

The addition of 46 mg/L of EGCG and of GSE significantly reduced the rate of heme-derived ^{55}Fe transport across the Caco-2 cell monolayer by $91.4 \pm 3.9\%$ and $91.4 \pm 4.4\%$, respectively. The question remained as to whether this inhibitory effect could be reversed by dietary ascorbic acid. The effect of 100 $\mu\text{mol/L}$ ascorbic acid on the EGCG- and GSE-mediated inhibition of heme iron absorption was investigated. The addition of ascorbic acid

did not counteract the inhibitory effects of EGCG and GSE on heme-derived ^{55}Fe transport across the Caco-2 cell monolayer (Figure 1). The rate of heme-derived ^{55}Fe transport was decreased to $91.1 \pm 1.9\%$ and $91.8 \pm 3.2\%$ in the cells treated with EGCG and GSE in the presence of ascorbic acid, respectively, compared with the control. These inhibited transport rates were similar to those for cells treated with EGCG and GSE alone. The addition of 100 $\mu\text{mol/L}$ ascorbic acid also had no effect on the heme- ^{55}Fe transport across the cell monolayer in the presence of EGCG and GSE (Figure 1).

Dose-dependent inhibitory effects of EGCG, GSE and GT

Because EGCG and GSE almost completely blocked intestinal heme- ^{55}Fe absorption at the concentration of 46 mg/L, the effects of lower concentrations of these polyphenols on heme-iron absorption were tested. Green tea is the major natural source of EGCG, so the effect of GT on heme- ^{55}Fe absorption was also examined. All of the tested polyphenolic compounds (EGCG, GSE and GT) exerted a dose-dependent inhibitory effect on heme- ^{55}Fe absorption across the Caco-2 cell monolayer, with half maximal inhibitory concentration (IC_{50}) values (95% confidence interval) of 3.6 (2.0–6.6), 3.0 (1.9–4.7) and 5.1 (4.0–6.5) mg/L, respectively, when 1 $\mu\text{mol/L}$ heme was applied to the system.

The treatment of EGCG at a lower concentration (4.6 mg/L) also significantly ($P < 0.05$) decreased the transepithelial transport of heme-derived ^{55}Fe to $28.1 \pm 5.2\%$ of the control, following a 7 h incubation time (Figure 2A). The addition of EGCG, even at a very low concentration (0.46 mg/L), significantly ($P < 0.05$) reduced the transport of heme-derived ^{55}Fe across the Caco-2 cell monolayer, and the amount of ^{55}Fe transferred from the apical to the basolateral compartment was $53.1 \pm 12.6\%$ of the control. The quantities of heme-derived ^{55}Fe transport across the cell monolayer increased linearly over the 7 h time course. Treatment by GSE at the lower concentrations of 4.6 and 0.46 mg/L significantly ($P < 0.05$) decreased the transepithelial transport of heme-derived ^{55}Fe to $34.4 \pm 10.6\%$ and $77.3 \pm 7.6\%$ of the control, respectively, during a 7 h incubation (Figure 2B). As indicated by the EGCG and GSE results, the addition of 46 mg/L GT also markedly inhibited the transepithelial transport of heme-derived ^{55}Fe across the cell monolayer to $10.1 \pm 2.5\%$ of the control (Figure 2C). The amounts of ^{55}Fe transported from the apical into the basolateral chamber were significantly ($P < 0.05$) reduced to 42.3 ± 6.2 and $70.5 \pm 2.2\%$ of the control by the addition of 4.6 and 0.46 mg/L of GT, respectively, during a 7 h incubation.

Concentrations of EGCG, GSE and GT as low as 0.46 mg/L inhibited ^{55}Fe transport across the cell monolayer when a low concentration (0.5 $\mu\text{mol/L}$) of ^{55}Fe -heme was added; these findings were also true when a higher concentration (5 $\mu\text{mol/L}$) was added. The transepithelial electrical resistance was not altered by the addition of EGCG, GSE, or GT, confirming the integrity of the Caco-2 cell monolayer under the conditions of the different treatments. The total cellular protein levels were not affected by treatments during the 7 h incubation.

Counteracting the effects of ascorbic acid on dietary polyphenols in heme-iron absorption

The next study was conducted to examine whether the regular consumption of ascorbic acid was capable of alleviating the inhibitory effect of lower concentrations of EGCG, GSE and GT on heme iron absorption. The inhibitory effect of 0.46 mg/L EGCG on the transepithelial transport of heme- ^{55}Fe was completely reversed by ascorbic acid, and the amounts of ^{55}Fe transported across the cell monolayer were 28% higher ($P < 0.05$) relative to the control (Figure 3A). In contrast, ascorbic acid was not able to counteract the negative impact of a higher concentration of EGCG (4.6 mg/L) on heme iron transport, although the level of inhibition was alleviated. The amount of ^{55}Fe transported across the cell monolayer was decreased by $35.7 \pm 2.0\%$ in cells treated with 4.6 mg/L EGCG plus ascorbic acid

(Figure 3A) but by $71.9 \pm 5.2\%$ in cells treated with 4.6 mg/L EGCG alone (Figure 2A), compared with control, over a 7 h time course.

Similarly, the inhibited heme ^{55}Fe transport by 0.46 mg/L GSE was completely reversed by ascorbic acid and was even enhanced above the control level. The amount of transported ^{55}Fe into the basolateral chamber was 41% higher ($P < 0.05$) than that of the control during the 7 h incubation (Figure 3B). The transepithelial ^{55}Fe transport was decreased by $59.2 \pm 2.0\%$ in the presence of 4.6 mg/L GSE alone (Figure 2B), but ascorbic acid significantly alleviated GSE's inhibitory action, and ^{55}Fe transport was only decreased by $24.5 \pm 4.0\%$ compared with the control (Figure 3B).

Ascorbic acid also completely reversed the inhibitory effect of 0.46 mg/L GT on heme iron absorption. The transepithelial transport of ^{55}Fe was enhanced by $48 \pm 0.4\%$ by ascorbic acid in the presence of 0.46 mg/L GT, compared with the control, during a 7 h incubation (Figure 3C). The addition of ascorbic acid was not able to reverse the inhibitory effect 4.6 mg/L GT on heme- ^{55}Fe transport, although the negative effect was mitigated. The addition of ascorbic acid, however, did not alter the inhibitory effect of 46 mg/L GT, as shown for EGCG and GSE (Figure 1B). Surprisingly, ascorbic acid itself significantly enhanced the amounts of ^{55}Fe transported into the basolateral chamber, by $97.9 \pm 25.3\%$ compared with the control. However, the apical uptake of heme- ^{55}Fe was not changed by ascorbic acid. As demonstrated from our ferrozine-based colorimetric assay, free iron was not detected in the apical uptake solution containing heme- ^{55}Fe , indicating that ^{55}Fe was added as heme- ^{55}Fe rather than as free ^{55}Fe during the iron transport study. Most of the ^{55}Fe (>97%) transported from the apical to the basolateral compartment across the cell monolayer was heme free iron.

Effects of ascorbic acid and polyphenols on the expression of iron relevant proteins and HO activity in Caco-2 cells

To investigate the possible mechanisms by which polyphenols and ascorbic acid modulate heme iron absorption, the expression of proteins involved in intestinal heme iron metabolism and basolateral iron transport and total HO activity were assessed. The expression of HO-1 and -2 proteins was not changed by ascorbic acid, EGCG, GSE, GT or by the cocktail of ascorbic acid and each of these polyphenols. Similarly, the expression of other proteins involved in iron metabolism and basolateral iron transport was not modulated by ascorbic acid and polyphenolic compounds (Figure 4). The total HO activity detected in the control cell culture was 0.77 ± 0.03 nmole bilirubin/min/mg protein, and was not affected by ascorbic acid and/or by polyphenolic compounds (Figure 5).

Discussion

Dietary heme-iron is principally provided by meat, blood-derived products and other animal tissues. It is well known that heme-iron absorption is relatively unaffected by other dietary factors that are common inhibitors of mineral absorption, such as phytates and fiber (Torre et al., 1991). The best known dietary factors that affect intestinal heme iron absorption are meat and calcium (Lopez and Martos, 2004). Heme iron absorption is increased by the presence of meat (Carpenter and Mahoney, 1992). In contrast, calcium is known to inhibit heme iron absorption in the same fashion that it inhibits non-heme iron at high concentrations (Hallberg et al., 1991). Although most research on the relationship between dietary factors and iron absorption has focused on non-heme iron, few studies have been conducted on heme iron.

An early study indicated that heme iron absorption decreased due to the consumption of tea in humans (Disler et al., 1975), and this finding was confirmed by our recent study (Ma et

al., 2010). We previously reported that dietary polyphenolic compounds (such as EGCG, a major polyphenolic compound in green tea, and GSE) markedly inhibited heme iron absorption when polyphenolic compounds were added at the concentration of 46 mg/L. Because many dietary factors modulate iron absorption in a dose-dependent manner (Hallberg et al., 1989; Tuntawiroon et al., 1991), we next investigated whether these selected bioactive polyphenolic compounds retained the ability to reduce heme iron absorption when added at lower concentrations. Our current data clearly indicated that EGCG and GSE inhibit heme iron absorption in a dose-dependent manner in human intestinal cells (Figure 2). Similarly, GT, the natural source of EGCG, also significantly decreased heme iron absorption in a dose-dependent way. Even at very low concentrations (0.46 mg/L), EGCG, GSE and GT significantly reduced heme iron transport across the cell monolayer during a 7 h transport assay. According to the IC₅₀ values, the transepithelial heme iron transport across the cell monolayer can be reduced 50% by 3.6, 3.0 and 5.1 mg/L of EGCG, GSE and GT, respectively.

Because the inhibitory effects of dietary factors on iron absorption can be offset or reversed by ascorbic acid, the most prominent dietary factor that enhances iron absorption, we next examined whether the inhibitory action of polyphenols on heme iron absorption can be counteracted by ascorbic acid. The addition of 100 µmol/L ascorbic acid completely reversed the inhibitory action of dietary polyphenols on heme iron absorption when the polyphenolic compounds were added at 0.46 mg/L. When the concentrations of polyphenols were increased to 4.6 mg/L, ascorbic acid was not able to counteract the inhibitory action of polyphenols on heme iron absorption, although the inhibition was reduced. However, ascorbic acid failed to have any positive effect on heme iron absorption when the polyphenols were added at a high (but still within physiological) level of 46 mg/L. These results imply that, while the inhibitory effect of low concentrations of bioactive polyphenols on heme iron absorption can be easily counteracted by ascorbic acid, the inhibitory action of high concentrations of polyphenolic compounds cannot be offset by regular consumption of dietary ascorbic acid.

Because the addition of ascorbic acid enhanced heme iron absorption above the control in the presence of the low level of polyphenolic compounds, we investigated the effect of ascorbic acid on heme iron absorption in the absence of dietary polyphenols. We found that ascorbic acid markedly enhanced heme iron transport across the cell monolayer without altering the apical uptake of heme. The applied ⁵⁵Fe- labeled heme remained intact because the free iron was not detected in solution containing heme-⁵⁵Fe. This finding is similar to a previous study on the effect of soy protein on heme iron absorption. Lynch et al. (1985) showed a significant increase in heme iron absorption by soy protein in human subjects (Lynch et al., 1985). However, the mechanism of the soy protein-mediated increase of heme iron absorption still remains to be explored.

To examine the possible mechanism by which ascorbic acid enhances heme iron absorption, we initially analyzed the expression of proteins involved in heme iron absorption and metabolism. Because these test solutions modulate heme transport across the cell monolayer without altering the apical heme uptake, we assessed the proteins involved in heme splitting, cellular iron metabolism and basolateral iron transport. Our western blot analysis data indicated that neither ascorbic acid nor selected polyphenolic compounds changed the expression of proteins involved in heme iron absorption and metabolism.

After heme enters the cell across the apical membrane of the enterocyte, it is then degraded by HOs to release ferrous iron and bilirubin, and the released iron enters into the soluble cytoplasmic pool of the enterocyte (Raffin et al., 1974). It is likely that, after heme iron is disassembled by HOs, the liberated iron enters the same storage and export pathways as

does non-heme iron. Duodenal HO activity was previously proposed as a limiting factor for heme iron absorption and shown to be linearly associated with heme iron absorption (Raffin et al., 1974; Wheby and Spyker, 1981). Therefore, to determine whether ascorbic acid and/or polyphenols modulate heme iron absorption by affecting the release of iron from heme, we assessed HO activity in cells treated with ascorbic acid and/or polyphenolic compounds. In the conventional HO assay, enzyme activity is measured by the rate of bilirubin formation, as iron release from heme is linearly associated with production of bilirubin (Raffin et al., 1974). As indicated by Figure 5, HO activity was not changed by ascorbic acid and polyphenolic compounds, suggesting that the addition of ascorbic acid and polyphenols modulated heme iron absorption without changing heme splitting in Caco-2 cells.

Our current data clearly indicate that ascorbic acid enhances heme iron absorption across the cell monolayer without modulating the apical heme uptake, indicating that ascorbic acid may affect cellular or basolateral events that increase heme-derived free iron export. The addition of ascorbic acid may enhance heme iron absorption by modulating several different steps in the cell, such as 1) by increasing the release of iron from heme, 2) by inducing basolateral iron export through an increase in FPN-1 expression, and 3) by facilitating the transfer of iron to the basolateral membrane. Because our western blot analysis data (Figure 4) and HO activity data (Figure 5) do not support the first two possible mechanisms, it is possible that ascorbic acid may increase the available iron pool for basolateral iron transporter or improve iron transport to the basolateral membrane.

It is believed that ascorbic acid mainly enhances non-heme iron absorption by reducing ferric to ferrous iron, a substrate of DMT-1 in the gastrointestinal lumen, and many studies have confirmed this conclusion (Raja et al., 1992; Han et al., 1995). A study (Han et al., 1995) previously demonstrated that ascorbic acid enhanced non-heme iron absorption by reducing ferric to ferrous iron and then increasing the apical iron uptake. However, the iron transported across the basolateral membrane was also enhanced (Han et al., 1995). Although the transepithelial iron transport across the Caco-2 cell monolayer was increased by 3.5-fold, the apical iron uptake was only elevated less than 2-fold compared with the control, indicating that ascorbic acid enhanced not only the apical uptake but also the basolateral transport. It is clear that ascorbic acid increases the apical uptake of non-heme iron, but it is unknown how ascorbic acid enhances the basolateral transport of iron. One proposed explanation is that ascorbic acid increases the basolateral iron transport by increasing the assimilation of iron into the cell (Han et al., 1995). In the current study, ascorbic acid enhanced the transepithelial iron transport without changing the apical heme uptake, HO activity or the level of FPN-1, suggesting that ascorbic acid may facilitate iron transfer to the basolateral membrane, leaving more free ⁵⁵Fe available for the iron exporter FPN-1. Together, our results indicate that small amounts of polyphenolic compounds in foods are capable of reducing heme iron transport across the intestinal enterocyte. However, these inhibitory effects of dietary polyphenolic compounds on heme iron absorption can be offset by ascorbic acid, and they can possibly be avoided by decreasing consumption of polyphenols while simultaneously consuming ascorbic acid.

Conclusions

Bioactive dietary polyphenols (including EGCG, GSE and GT) inhibit heme-iron absorption in a dose-dependent manner in human intestinal Caco-2 cells. Ascorbic acid cannot offset the inhibitory effects of high concentrations (46 mg/L) of polyphenols but can reverse the effects of lower concentrations of polyphenols.

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Figure 1.

Ascorbic acid does not reverse the inhibitory effects of EGCG and GSE on heme iron absorption. A: Transepithelial transport of heme-derived ⁵⁵Fe during 7 h incubation. Values are means $\pm$ SEM, n = 6. Means at a time without a common letter differ, P < 0.05. Within a treatment, means without a common symbol differ, P < 0.05. B: Relative amounts of ⁵⁵Fe transported from the apical (AP) to the basolateral (BL) chamber for 7 h incubation. Data are means $\pm$ SEM, n = 6. Means without a common letter differ, P < 0.05. VC; vitamin C.

Figure 2.

Polyphenols inhibit the transepithelial transport of heme-derived ^{55}Fe in a dose-dependent manner in Caco-2 cells. A: EGCG, B: GSE, and C: GT. Values are means $\pm$ SEM, $n = 6$. Means of different doses at the same time point without a common letter differ, $p < 0.05$. Means of different time points for the same treatment without a common symbol differ, $p < 0.05$.

Figure 3.

Ascorbic acid can reverse or alleviate the inhibitory effects of polyphenols at concentrations of ≤ 4.6 mg/L on heme iron absorption in human intestinal cells. A: EGCG, B: GSE and C:

GT. Ascorbic acid was added at 100 $\mu\text{mol/L}$. Values are means $\pm$ SEM, $n = 6$. Means of different doses at the same time point without a common letter differ, $p < 0.05$. Means of different time points for the same treatment without a common symbol differ, $p < 0.05$.

Figure 4. Effects of ascorbic acid, EGCG, GSE and GT on the expression of proteins involved in intestinal heme-iron absorption and metabolism. Representative western blots of HO-1, HO-2, FPN-1, Heph, TfR1, Ferritin (Ft) protein expression from Caco-2 cells treated with 100 μ mol/L ascorbic acid (vitamin C;VC), 46 mg/L EGCG, GSE and GT for 7 h. Calnexin was used as a loading control.

Figure 5. Effects of polyphenols and ascorbic acid on HO activity. Cells were treated with 46 mg/L EGCG, GSE, GT and 100 μ mol/L ascorbic acid for 7 h. Values are means $\pm$ SEM, n = 6. No significant differences were found in HO activity.