Regular Article

Effect of Epigallocatechin Gallate on Drug Transport Mediated by the Proton-coupled Folate Transporter

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Summary: Folic acid (FA) is a water-soluble vitamin, and orally ingested FA is absorbed from the small intestine by the proton-coupled folate transporter (PCFT). In the present study, we investigated whether epigallocatechin gallate (EGCG), one of the tea catechins, affects the transport of FA by PCFT. EGCG inhibited the uptake of FA into Caco-2 cells and human PCFT-expressing HEK293 cells (PCFT-HEK293 cells). The initial rate of uptake of FA into PCFT-HEK293 cells followed Michaelis–Menten kinetics \( (K_m = 1.9 \, \mu M) \). Dixon plots revealed that PCFT-mediated FA uptake was competitively inhibited by EGCG \( (K_i \approx 9 \, \mu M) \). The uptake of the PCFT substrate methotrexate (MTX) was competitively inhibited by EGCG as well \( (K_i \approx 15 \, \mu M) \). In conclusion, it is suggested that when FA or MTX is ingested with tea, it is likely that the intestinal absorption of these compounds by PCFT is inhibited, which could result in insufficient efficacy.

Keywords: epigallocatechin gallate; folic acid; PCFT; methotrexate; catechin

Introduction

The water-soluble vitamin folic acid (FA) functions as a coenzyme in one-carbon transfer reactions in nucleic acid biosynthesis and amino acid metabolism and plays an important role in maintaining homeostasis. Because folates are not biosynthesized by mammals, they are obtained from nutrients. Folates exist as anions under physiological pH, and their permeation of cell membranes by passive diffusion is expected to be extremely low. Indeed, specific transporters mediate the permeation of folates through cell membranes. Folates are substrates of the reduced folate carrier (RFC, SLC19A1) and the proton-coupled folate transporter (PCFT, SLC46A1), which are expressed by various mammalian cell types. The cDNAs of RFC have been molecularly cloned from mouse, hamster, and human cells, and the transport characteristics of RFCs have been clarified. RFC transports only reduced folates, and maximal folate transport occurs at neutral pH. In contrast, PCFT was originally identified as the heme carrier protein (HCP), and it was subsequently revealed that its substrates are folates. PCFT is expressed by epithelial cells of the small intestine and is involved in the absorption of reduced and oxidized folates using an H\(^+\) gradient as a driving force. Because the pH at the surface of epithelial cells is acidic, PCFT is considered to play a major role in the intestinal absorption of folates.

Hereditary folate malabsorption (HFM) is a rare autosomal recessive disorder characterized by the signs of a folate deficiency that appear within several months after birth. Infants with HFM develop anemia, diarrhea, immune deficiency, peripheral neuropathy, and seizures as well as failure to thrive. Studies on patients with HFM revealed loss of PCFT function due to mutations in the gene encoding PCFT (SLC46A1). Loss of PCFT function leads to decreased intestinal absorption of folate and reduced folate transport into the central nervous system. These findings demonstrate the important role of PCFT in intestinal absorption and distribution of folates into the brain.

When FA is ingested with green or black tea, its maximum serum concentration (\( C_{\text{max}} \)) and AUC decrease by approximately 40% and 20% compared with the control (ingested with water), respectively. Myricetin, isoxanthohumol, and epigallocatechin gallate (EGCG) are phenolic compounds present in wine, beer, or tea that inhibit FA uptake by Caco-2 cells. Moreover, FA uptake by Caco-2 cells is inhibited by black and green tea extracts. EGCG is the major catechin contained in green and black teas.

The present study confirms the inhibitory effect of EGCG on FA uptake by Caco-2 cells and reveals the effect of EGCG on the uptake of FA by human PCFT-expressing HEK293 cells (PCFT-HEK293 cells). Finally, we present data on the influence of EGCG on the uptake of the PCFT substrate methotrexate (MTX).

Materials and Methods

Materials: Folic acid (FA) and methotrexate (MTX) were purchased from Nacalai Tesque (Kyoto, Japan). Epigallocatechin gallate was purchased from Enzo Life Sciences (Farmingdale, NY). HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid), and MES (2-morpholinoethanesulfonic acid, monohydrate)
were purchased from Nacalai Tesque. [3H]FA (specific activity, 16.4 Ci/mmol) and [3H]MTX (specific activity, 15.7 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA) and used without further purification. All other chemicals were of the highest grade available.

**Cell culture:** Caco-2 cells (passage 18) were obtained from American Type Culture Collection (Manassas, VA). Culture conditions were the same as those reported previously. Cells between the 25th and 40th passages were used in the present study and were cultured in multiwell plates at 1.0 × 10^5 cells per well for 7 days before being used in the uptake studies.

Stably transfected HEK293 cells expressing human PCFT (PCFT-HEK293 cells) and control cells (Mock) were generated as described previously and maintained at 37°C in a humidified atmosphere containing 5% CO2. The culture medium consisted of Dulbecco’s modified Eagle’s medium (DMEM), 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum. PCFT-HEK293 cells and HEK293-Mock cells were grown in DMEM containing 100 U/mL penicillin, 100 µg/mL streptomycin, 600 µg/mL G418 sulfate, and 10% fetal bovine serum. Cells were seeded in multiwell plates coated with poly-d-lysine at a density of 1.0 × 10^5 cells per well.

**Uptake study:** Hanks’ balanced salt solution (HBSS, 145 mM NaCl, 3 mM KCl, 1 mM CaCl2, 0.5 mM MgCl2) containing 5 mM d-glucose and 5 mM MES (pH 6.0) was used as the uptake medium. HBSS containing 5 mM d-glucose and 5 mM HEPES (pH 7.4) was used as the rinse medium. To measure uptake, Caco-2, PCFT-HEK293, and HEK293-Mock cells in multiwell plates were rinsed twice and incubated with the rinse medium (pH 7.4) for 10 min at 37°C. Uptake was initiated by adding 0.5 mL of the drug solution, which was aspirated to terminate uptake. The cells were rinsed twice with ice-cold rinse medium (pH 7.4) and then lysed with 1 mL of 0.1% Triton® X-100. To determine the uptake amount of [3H]FA or [3H]MTX with or without EGCG was used as the drug solution, which was aspirated to terminate uptake. The cells were rinsed twice with ice-cold rinse medium (pH 7.4) and then lysed with 1 mL of 0.1% Triton® X-100. To determine the uptake amount of [3H]FA or [3H]MTX, a 0.8 mL aliquot of the cell lysate was transferred to a scintillation vial containing 5 mL of Clear-sol I (Nacalai Tesque), and the radioactivity was measured by using a liquid scintillation counting. Drugs and inhibitors were dissolved in dimethylsulfoxide (DMSO) at a final DMSO concentration ≤1%. Adsorption of the drug to Caco-2 cells was measured in the same manner as described above, except that the drug solution was removed immediately after it was added to the plate. The amount incorporated was subtracted to obtain the net uptake.

**Protein assay:** Caco-2, PCFT-HEK293, and HEK293-Mock cells were dissolved in 1 mL of 0.1% Triton X-100, and protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as the standard.

**Data analysis:** To calculate the kinetic parameters for FA uptake, the data were fit to the following equation using a nonlinear least-squares method.

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v = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]}
\]

where \(v\) is the carrier-mediated initial uptake rate, \(V_{\text{max}}\) is the maximum uptake rate, \(K_m\) is the Michaelis constant, and \([S]\) is the initial concentration of the drug.

**Statistical analysis:** Statistical analysis was conducted using the Student’s t test.

**Results**

**Uptake of FA by Caco-2 and PCFT-HEK293 cells:** The uptake of 0.02 µM [3H]FA into Caco-2 cells was measured for 5 min at extracellular pH values ranging between 6.0 and 7.4 (Fig. 1A). Uptake was highest at pH 6.0 and decreased with an increase in the extracellular pH. The time course of uptake of 0.02 µM FA into Caco-2 cells in the presence of a H+ gradient (pH 6.0) is shown in Figure 1B. Because the amount of TA taken up into the cells increased linearly for at least 15 min, the initial uptake rate was calculated from the uptake amount at 2 min in the following analyses. The inhibitory effects of EGCG as a function of its concentration on the uptake of 0.02 µM [3H]FA into Caco-2 cells are shown in Figure 2.

**Figure 3A** shows the pH-dependent uptake of 0.02 µM [3H]FA into PCFT-HEK293 and Mock cells. Uptake was measured at extracellular pH levels ranging between 6.0 and 7.4 for 2 min. Uptake was significantly higher into PCFT-HEK293 cells compared with the Mock cells. Uptake mediated by PCFT (uptake into PCFT-HEK293 cells minus that into Mock cells) was the highest at pH 6.0 and decreased with increased extracellular pH, which was similar to that for Caco-2 cells (Fig. A). **Figure 3B** shows the time course of uptake of 0.02 µM [3H]FA into PCFT-HEK293 and Mock cells at extracellular pH 6.0. Uptake into PCFT-HEK293 cells was significantly higher compared with the Mock cells. The initial uptake rate was calculated from the uptake amount at 1 min (Fig. 3B) in the analyses that follow.

The initial uptake rates of 0.02–10 µM [3H]FA were measured in the presence of a H+ gradient (pH 6.0) in PCFT-HEK293 and

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![Fig. 1. pH-dependent uptake of 0.02 µM FA into Caco-2 cells (A); time course of the uptake of FA at 0.02 µM into Caco-2 cells at pH 6.0 (B)](image)

Each point represents the mean ± S.D. of three independent determinations.

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Mock cells. PCFT-mediated uptake was calculated by subtracting the uptake values of Mock cells from those of PCFT-HEK293 cells (Fig. 4). The uptake parameters calculated according to Eq. (1) were as follows: $K_m = 1.90 \pm 0.59 \mu M$, $V_{max} = 20.1 \pm 4.2 \, \text{pmol/min/mg protein}$ [mean $\pm$ standard deviation (S.D.), $n = 3$]. Figure 5 shows the inhibitory effects of EGCG as a function of its concentration on the uptake of 0.02 $\mu M$ [3H]FA into PCFT-HEK293 cells, which were comparable to those for Caco-2 cells (Fig. 2).

The initial uptake rates of 0.02, 0.034, and 0.1 $\mu M$ [3H]FA into PCFT-HEK293 and Mock cells were measured in the presence of 0, 5, and 10 $\mu M$ of EGCG. PCFT-mediated uptake was calculated, and the data were analyzed using a Dixon plot (Fig. 6), which shows that EGCG competitively inhibited [3H]FA uptake with a $K_i$ value of 9.15 $\mu M$.

Inhibitory effect of EGCG on the uptake of MTX: Figure 7 shows the inhibitory effect of EGCG on the uptake of 0.02 $\mu M$ [3H]MTX into Caco-2 (A) and PCFT-HEK293 cells (B). The uptake of MTX was inhibited with increasing EGCG concentrations. The initial uptake rates of 0.25, 0.4, and 1 $\mu M$ of [3H]-MTX into PCFT-HEK293 and Mock cells were measured in the presence of 0, 10 and 20 $\mu M$ of EGCG. PCFT-mediated uptake was calculated, and the data were analyzed using a Dixon plot (Fig. 8), which shows that EGCG competitively inhibited MTX uptake with a $K_i$ value of 15.4 $\mu M$.

Discussion

The proton-coupled folate transporter (PCFT, SLC46A1), which is a member of the SLC family, transports reduced and oxidized
folates using a H⁺ gradient as a driving force.⁷,⁸ Because the pH at the surface of the apical membrane of intestinal epithelial cells is acidic, several transporters transport substrates actively across the cell membrane utilizing the pH gradient. PCFT is one of these transporters, is expressed in the apical membrane of intestinal epithelial cells, and plays a major role in the intestinal absorption of folates.⁸

Hereditary folate malabsorption (HFM) is characterized by folate deficiency due to impaired intestinal absorption of folic acid (FA).¹⁷ The association of HFM with a functional loss of PCFT caused by SLC46A1 mutations¹⁸,¹⁹ strongly supports the conclusion that PCFT plays a major role in the intestinal absorption of folates.²⁰

MTX (L-amethopterin) is an antineoplastic and antirheumatic drug that is administered orally. When an equal dose of L-amethopterin (MTX) or its enantiomer (D-amethopterin) is orally administered to humans, the absorption of L-amethopterin is approximately 40 times greater than that with D-amethopterin.²⁷ The marked difference in intestinal absorption between the enantiomers is due to their stereoselective transport by PCFT.²⁵ If L-amethopterin and D-amethopterin were absorbed by passive diffusion, their intestinal absorption would be similar. These observations strongly support the conclusion that MTX is absorbed from the small intestine mainly via transport by PCFT.

The Caco-2 cell line is widely used as a model of small-intestine epithelial cells. Many influx and efflux transporters that belong to the SLC (solute carrier) transporter family and the ABC (ATP-binding cassette) transporter are expressed in Caco-2 cells, including RFC and PCFT.²⁸-³¹ We measured the uptake of L- and D-MTX into Caco-2 cells previously and found that L-MTX uptake is at least 7 times greater than that of D-MTX.³²

The pH-dependent uptake of 0.02 µM [3H]FA was measured at extracellular pH values from 6.0 to 7.4. Uptake was highest at pH 6.0 and decreased with an increase in extracellular pH that was similar for PCFT-HEK293 and Caco-2 cells (Figs. 1A and 3A), and our findings for PCFT-HEK293 cells are consistent with those of others.⁷,³³ Moreover, the Kₘ value (1.90 µM) for FA uptake into PCFT-HEK293 cells (Fig. 3) is similar to those reported previously (1.5 µM in Xenopus oocytes injected with human PCFT cRNA⁷ and 1.67 µM in HEK293 cells transfected with human PCFT cDNA).³³ These results indicate that FA uptake at acidic pH reflects that of PCFT and not RFC.

FA uptake into Caco-2 cells was reduced in the presence of increasing concentrations of EGCG. We reported previously that FA uptake into Caco-2 cells is significantly inhibited by 2 µM and 100 µM unlabeled FA and MTX.³⁴ In contrast, FA uptake is not affected by thiamin monophosphate (TMP) or thiamin pyrophos-
phate (TPP), which inhibit RFC. In the presence of 20 µM TMP or TPP, FA uptake into Caco-2 cells is 119 ± 18% or 102 ± 6.8% (mean ± S.D., n = 3) of the control, respectively. These results support the conclusion that FA was transported into Caco-2 cells by PCFT, which was inhibited by EGCG.

EGCG exerted similar inhibitory effects on PCFT-mediated FA uptake into PCF-HEK293 or Caco-2 cells. In our preliminary study, PCFT-mediated FA uptake decreased by 50% or 66% compared with the control in the presence of 2 µM unlabeled FA or MTX, respectively. In contrast, PCFT-mediated FA uptake was not inhibited by TMP or TPP. The PCFT-mediated uptake of FA in the presence of 20 µM TMP or TPP was 87 ± 4.4% or 105 ± 1.5% (mean ± S.D., n = 3) of the control, respectively. The Dixon plot showed that FA uptake was competitively inhibited by EGCG (K_i = 9.15 µM) (Fig. 6). Other studies report that EGCG inhibits FA uptake into Caco-2 cells with IC_{50} values of 7.7 µM and 34.8 µM.31,22

The catechins present in highest abundance in various teas are epigallocatechin, catechin, epicatechin, EGCG, and epicatechin gallate. Among them, EGCG is the most abundant in every brand of tea. Alemdaroglu et al.22 dissolved 0.3 g of green tea extract 250 mL water, and FA was ingested with the extract or water (control). The maximum concentration (C_{max}) and AUC decreased to approximately 40% and 20% of the control, respectively, when FA was ingested with the solution containing green tea extracts. Green tea extracts contain 208 µmol/g EGCG.22 Assuming 1.92 L as the apparent volume of intestinal fluid, the concentration of EGCG is 32 µM.39 Therefore, the concentration of EGCG would be sufficient to inhibit FA absorption by PCFT, because the K_i value of EGCG is 9.15 µM, as described above.

We reported previously that MTX is transported by PCFT with a K_m = 4.98 µM.25 Other studies show that EGCG inhibits MTX uptake into Caco-2 cells with an IC_{50} = 10.1 µM. In the present study, EGCG competitively inhibited MTX uptake with a K_i = 15.4 µM (Fig. 8). Taken together, these data strongly indicate that the absorption of MTX will be reduced if it is consumed with green or black tea.

Transporters play an important role in the intestinal absorption of orally administered drugs. Interactions between drugs and between drugs, foods, and beverages are likely to occur in transporter-mediated absorption. For example, the plasma concentration of fexofenadine is significantly reduced when consumed with fruit juice.40 These findings suggest that the main inhibitors of fexofenadine absorption by the organic anion transporting polypeptide 2B1 (OATP2B1, SLCO2B1) are naringin, a component of grapefruit juice, and hesperidin, a component of orange juice.41,42

EGCG inhibits the activities of several transporters, including OATP1A1, OATP1B1, and OATP2B1.43 OATPs are involved in the transport of a variety of amphipathic drugs. For example, EGCG, but not other green tea catechins, inhibits the apical sodium dependent bile acid transporter (ASBT, SLCL0A2), which plays a role in the absorption of bile acid by the intestine.44 Moreover, EGCG decreases the export of mitoxantrone from the cell by inhibiting the breast cancer resistance protein (BCRP, ABCG2), which is an efflux transporter.45 Because EGCG inhibits influx and efflux transporters, transporter-mediated drug-beverage interactions could take place when a drug is taken with beverages containing EGCG.

The present study reveals for the first time that EGCG is a competitive inhibitor of FA and MTX transport mediated by PCFT. These findings suggest that the reported reduction of FA concentrations in plasma after its ingestion with green or black tea is caused by the inhibition of PCFT by EGCG. Therefore, we conclude that it is very likely that the absorption of orally administered MTX will be reduced if MTX is administered with beverages such as green tea or black tea that contain EGCG.

References

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