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Intestinal efflux transport kinetics of green tea catechins in Caco-2 monolayer model

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Abstract

The bioavailability of green tea catechins (GTCs), including epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC) is low in both animals and humans. The contribution of intestinal efflux to this low bioavailability has been suggested by previous studies. The objective of the present study was to investigate the kinetics of efflux transport of the four major GTCs in Caco-2 cell lines, to provide comparison on the efflux transport between each GTC. The basal-to-apical transport of each GTC at concentrations ranging from 15 to 265 μM was examined using the Caco-2 cell monolayer model. Transported amount of GTC was measured by high-performance liquid chromatography with electrochemical detection. Kinetic parameters, V_{max}, K_m and V_{max}/K_m were determined and compared among the four studied GTCs. The extent of basal-to-apical transport was, in descending order, EC > EGC > ECG ≈ EGCG. Kinetic studies indicated that active and saturable efflux transport of EC took place in Caco-2 cells, with a K_m of 131 μM, a V_{max} of 0.0249 nmol min cm^{-2} and an intrinsic clearance (V_{max}/K_m) of 0.19 μL min cm^{-2}. No saturation could be observed for the efflux transport of EGC, ECG and EGCG even at concentrations up to about 200 μM, which may be due to their low affinity towards the transporters at the concentration range studied. In conclusion, the extent of efflux transport of GTCs in Caco-2 cells was, in descending order, EC > EGC > ECG ≈ EGCG, which may reflect the order of elimination occurring in the intestine. The kinetic studies showed the importance of efflux transporters in basal-to-apical transport of EC and suggests their role in the limited oral bioavailability of EC.

Introduction

Tea (*Camellia sinensis*) is one of the most popular beverages in the world. Tea is classified into green tea, oo-long tea and black tea according to the degree of fermentation (unfermented, semi-fermented and fully fermented, respectively). Green tea is rich in polyphenols, flavan-3-ols being one of the major beneficial substances (Takehiko & Mujo 1997). In brewed green tea, the water-extractable material, which usually accounts for one-third of the tea leaves in dry weight, contains about 30% of flavan-3-ols (Balentine et al 1997). Among the major green tea catechin (GTC) components, epigallocatechin gallate (EGCG) is the richest in amount, followed by epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC) in the decreasing order of abundance (Takehiko & Mujo 1997). The chemical structures of the four major GTCs are shown in Figure 1.

The beneficial effects of green tea to human health are of great interest. Experimental and epidemiological data have demonstrated that the polyphenolic components present in green tea possess anticarcinogenic properties (Yang & Wang 1993) and cardiovascular protective effects (Hertog et al 1995; Geleijnse et al 1999; Yang et al 2004).

As the physiological effects of drugs are proportionally related to their concentrations in the blood, various groups have investigated the pharmacokinetics of GTCs. The oral bioavailability of GTCs has been demonstrated to be low in both rats (Chen et al 1997; Zhu et al 2000) and humans (Yang et al 1998; Chow et al 2001; Warden et al 2001). However, intestinal efflux rather than hepatic elimination was suggested to play a significant role in the pre-systemic elimination of orally administered GTCs (Cai et al 2002). Therefore, studies on intestinal absorption of GTCs are necessary to determine the contribution of intestinal efflux to the their low oral bioavailability.

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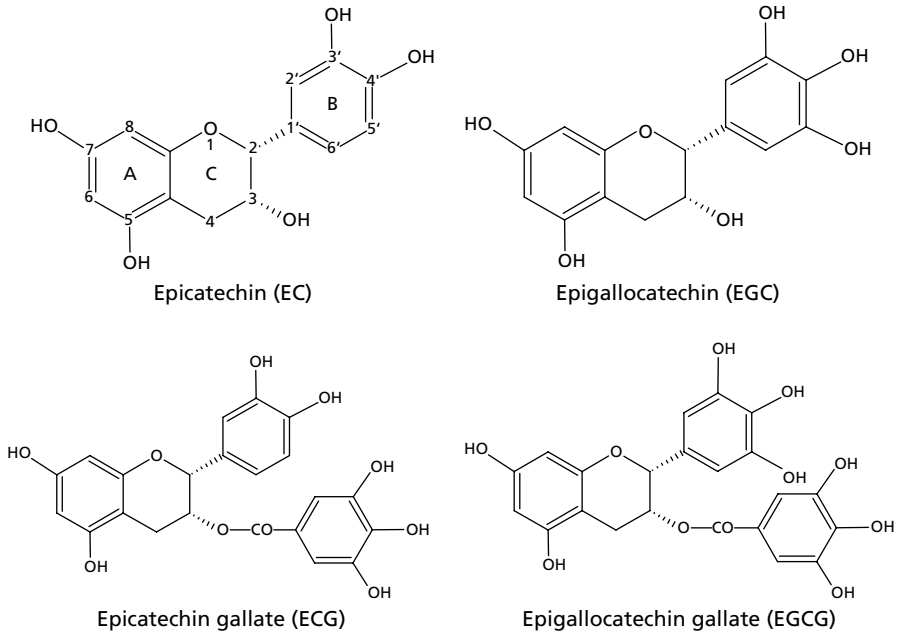

Figure 1 Structures of green tea catechins.

Previous studies using the Caco-2 cell monolayer model found that the four major GTCs (EC, EGC, ECG and EGCG) at a concentration of 50 μM demonstrated limited transepithelial absorption transport but extensive efflux mediated by multidrug resistance protein (MRP) rather than P-glycoprotein (Vaidyanathan & Walle 2001; Zhang et al 2004). Among the four GTCs, EC exhibited the most intensive intestinal efflux in the Caco-2 monolayer model. Moreover, investigation on the competition among the four major GTCs suggests that EC would compete with other GTCs for MRP (Zhang et al 2006). In the cellular uptake study of EGCG by Hong et al (2003), it was demonstrated that EGCG was transported by MRP1 and MRP2. In another cellular uptake study of ECG, the role of MRP1 and MRP2 in the transport of ECG was also demonstrated (Vaidyanathan & Walle 2003). Thus, previous observations suggest that MRPs are involved in the intestinal transport of GTCs. The differences in the structures of the four major GTCs may result in different extents of intestinal efflux. However, the intestinal efflux transport kinetics of these GTCs has not been reported. The present study aimed to study the kinetics of the intestinal efflux transport of the four major GTCs using the Caco-2 monolayer model.

Materials and Methods

Chemicals

(–)-EC, (–)-EGC, (–)-ECG, (–)-EGCG and phosphate buffer saline (PBS) tablets were purchased from Sigma Chemical Co., USA. Isoquercitrin, serving as internal standard for determining the concentration of EC and EGCG, was from Carl Roth, Germany. High-performance liquid chromatography (HPLC) grade methanol and acetonitrile were obtained from Labscan Asia Co. Ltd, Thailand. Other chemicals used were commercially available and of reagent grade.

Materials for cell culture

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 0.05% trypsin-ethylenediamineteraacetic acid, penicillin/streptomycin, and non-essential amino acids were all obtained from Gibco BRL, Life and Technologies, USA. Collagen type I and sodium pyruvate were bought from Sigma Chemical Co., USA. Caco-2 cells were obtained from the American Type Culture Collection. Six-well Transwell inserts (0.4-μm pore size, 4.71 cm^2, polycarbonate filter, Costar 3410) were purchased from Corning Costar Co., NY, USA.

Instruments

The HPLC system comprised a Waters 2690 separation module and a Waters 464 electrochemical detector was used for sample analysis. An ODS reversed-phase column (4.6 mm i.d. $\times$ 250 mm, 4.5 μm; Beckman, CA, USA) was used for separation.

Establishment of the Caco-2 cell monolayer model

Caco-2 cells were cultured as described previously (Zhang et al 2004, 2006). Briefly, DMEM supplemented with 10% FBS, 1% non-essential amino acids, 0.2% sodium pyruvate and 0.5% penicillin/streptomycin served as the basic medium. The Caco-2 cell suspension was seeded at a density of 3×10^5 cells/well in a 6-well Transwell insert coated with a collagen layer. The efflux transport experiments were conducted with

monolayers between 20 and 22 days in culture. Transepithelial electrical resistance (TEER) was used to check the integrity of the monolayers. Only inserts with TEER exceeding 940 Ω cm^2 (subtracting the background of the Transwell insert) were used for the transport study.

Preparation of GTC solutions for the efflux transport study

Individual GTC solutions were prepared by dissolving each GTC in PBS^+ transport buffer (pH 6.0) to give concentrations ranging from about 15 μM to 265 μM. PBS^+ was prepared by dissolving one PBS tablet in 200 mL distilled water containing 90 μL Ca^{2+} (2 μM) and 80 μL Mg^{2+} (1 μM) followed by adjusting the pH to 6.0.

Efflux transport study of GTCs in the Caco-2 cell monolayer model

The prepared Caco-2 cell inserts were rinsed twice with warm PBS^+ transport buffer and equilibrated at 37°C with PBS^+ transport buffer for 15 min before the transport experiment. Then, 1.5 mL blank PBS^+ was placed in the apical side (receiver chamber) and 2.6 mL of GTC solutions dissolved in PBS^+ at appropriate concentrations (ranging from 15 to 265 μM) was placed in the basolateral side (donor chamber). Samples (0.5 mL) were taken from the receiver chambers at different time intervals after loading (30, 60, 90 and 120 min). After each sampling, an identical volume of fresh PBS^+ was replaced in the receiver chambers to maintain a constant volume. Samples were also withdrawn from the donor chambers at the end of the experiment to determine recovery.

Sample treatment and analysis

Samples obtained from efflux transport studies were acidified with ascorbic acid solution, containing 0.6% ascorbic acid and 0.038% H_3PO_4 to reach pH 2.5 for stability considerations (Chen et al 2001; Zhu et al 2003). The ratio of sample to ascorbic acid solution was 5:1 (v/v). The samples were stored at −80°C until analysis.

A 200-μL sample was spiked with 20 μL of various internal standards and analysed as described previously (Zhang et al 2004). The HPLC system connected to the electrochemical detector with potential set at 1000 mV was used for the detection of GTCs. The ODS reversed-phase column was used for the separation. Each GTC was analysed using a separate mobile phase system. The detection limit was 5 ng mL^{-1} for all four GTC.

Calculation of recovery

In order to estimate the loss of compounds due to degradation, cell uptake or non-specific binding during the transport experiments, samples were withdrawn from the donor chambers at the end of the experiments to determine the recovery of the loading compounds. The recovery (%) of the compounds was calculated as follows: recovery (%) = (final amount of parent compound found in both donor and receiver chambers/initial amount of parent compound loaded in the donor chambers) × 100%.

Calculation of apparent permeability coefficient

The apparent permeability coefficient (P_{app}, cm s^{-1}) of each GTC in the efflux transport study was calculated according to the equation $P_{app} = [(dC/dt \times V)]/(A \times C)$, where dC/dt is the change in the drug concentration in the receiver chambers over time (μM s^{-1}); V is the volume of the solution in the receiver chambers (cm^3); A represents the membrane surface area (cm^2); and C is the initial loading concentration in the donor chambers (μM) (Vaidyanathan & Walle 2001).

Calculation of kinetic parameters

For kinetic studies of the efflux transport of the four major GTCs in the Caco-2 monolayer model, the kinetic parameters of V_{max} and K_m were calculated according to the Michaelis–Menten equation: $\upsilon_o = V_{max} \times C/(K_m + C)$, where υ_o is the rate of transport of substrates (nmol min cm^{-2}), C is the substrate concentration in the donor compartment (μM), V_{max} is the maximum transport rate (nmol min cm^{-2}) and K_m is the Michaelis constant (μM), which is equal to the substrate concentration at which the half-maximal transport rate is achieved. V_{max}/K_m (μL min cm^{-2}) implies the intrinsic clearance of GTCs in Caco-2 cell lines. The data were used for quantitative comparison of the efflux transport of each GTC.

Data analysis

Results are presented as mean ± s.e. of at least three experiments. For the kinetic studies of the efflux of GTCs, the data were fitted to the Michaelis–Menten equation by the non-linear least squares method to obtain estimates of the kinetic parameters. Statistical significant differences were evaluated by the Kruskal–Wallis test with a level of $P < 0.05$ considered significant.

Results

Efflux transport of GTCs in the Caco-2 cell monolayer model

Selection of the tested concentrations for each GTC was based on the compromise between the detection limits of the current analytical method and the concentrations that may cause physical damage to the Caco-2 cells. The concentrations of GTC used (15 to 263 μM) were believed to cause no physical damage to the Caco-2 monolayers as the net TEER values were found to be greater than 940 Ω cm^2 before and after the transport experiments. EC and EGC with loading concentrations greater than 300 μM were found to cause significant drops in TEER values to below 480 Ω cm^2, indicating that damage to the integrity of the monolayers may have occurred.

For each GTC at various loading concentrations, the amounts transported were all linear with time for up to 2 h. The recovery of all the GTCs was about 90% and was not concentration dependent. For each GTC, there were no significant differences in the recovery (%) values among various loading concentrations ($P > 0.05$). In addition, P_{app} values of the efflux transport of the four studied GTCs at loading concentrations of about 50 μM were calculated and compared. There were significant differences in P_{app} (basolateral to apical) values among the four GTCs ($P < 0.05$) in descending order: EC (P_{app} of $1.88 \pm 0.12 \times 10^{-6}$ cm s^{-1} at 42 μM) > EGC (P_{app} of $0.65 \pm 0.04 \times 10^{-6}$ cm s^{-1} at 50 μM) > ECG (P_{app} of $0.15 \pm 0.02 \times 10^{-6}$ cm s^{-1} at 53 μM) ~ EGCG (P_{app} of $0.13 \pm 0.01 \times 10^{-6}$ cm s^{-1} at 63 μM).

Kinetics of efflux transport of GTCs on Caco-2 cell lines

Figure 2 shows the relationships between efflux transport rates and the loading concentrations of the four GTCs. At low concentrations, efflux transport rates of EC increased as the loading concentration increased, followed by a plateau. EC showed a saturable secretory mechanism, which indicated the involvement of a carrier-mediated process (Shargel & Yu 1999). When efflux transport rates at different loading concentrations of EC were fitted to the Michaelis–Menten equation, K_m and V_{max} values were found to be 131.0 ± 8.9 μM and 0.0249 ± 0.0009 nmol min cm^{-2}, respectively. V_{max}/K_m, which implies the intrinsic clearance of EC in Caco-2 cell lines, was 0.19 μL min cm^{-2}.

Unlike EC, the efflux transport rates of EGC, ECG and EGCG increased in a concentration-dependent manner and did not reach a plateau over the tested concentration range. Hence, K_m and V_{max} could not be obtained. Assuming the substrate concentration was still far lower than the concentration that caused saturation of the transporters ($C << K_m$), the term C in the denominator of the Michaelis–Menten equation ($\upsilon_o = V_{max} \times C/(K_m + C)$) could be dropped to give a simplified equation: $\upsilon_o = V_{max} \times C/K_m$. This equation can be rearranged to give $V_{max}/K_m = \upsilon_o/C$. Thus, the V_{max}/K_m value could be found from the slope of the plot of rates of transport against loading concentrations (Tracy et al 2003). The intrinsic clearance of EGC, ECG and EGCG was estimated to be 6.899×10^{-2}, 2.303×10^{-2} and 1.364×10^{-2} μL min cm^{-2}, respectively. Hence, the intrinsic clearance of GTCs was, in descending order: EC > EGC > ECG > EGCG.

Discussion

Our previous studies indicated that the four GTCs have very limited apical-to-basal transepithelial transport in Caco-2 monolayers, with P_{app} values similar to that of the paracellular marker (Zhang et al 2004). The absorption transport of the four GTCs is mainly mediated by passive diffusion through the paracellular pathway, whereas their more significant basal-to-apical efflux involves active transport. Therefore, only the efflux transport kinetics of the four GTCs were investigated in the current study.

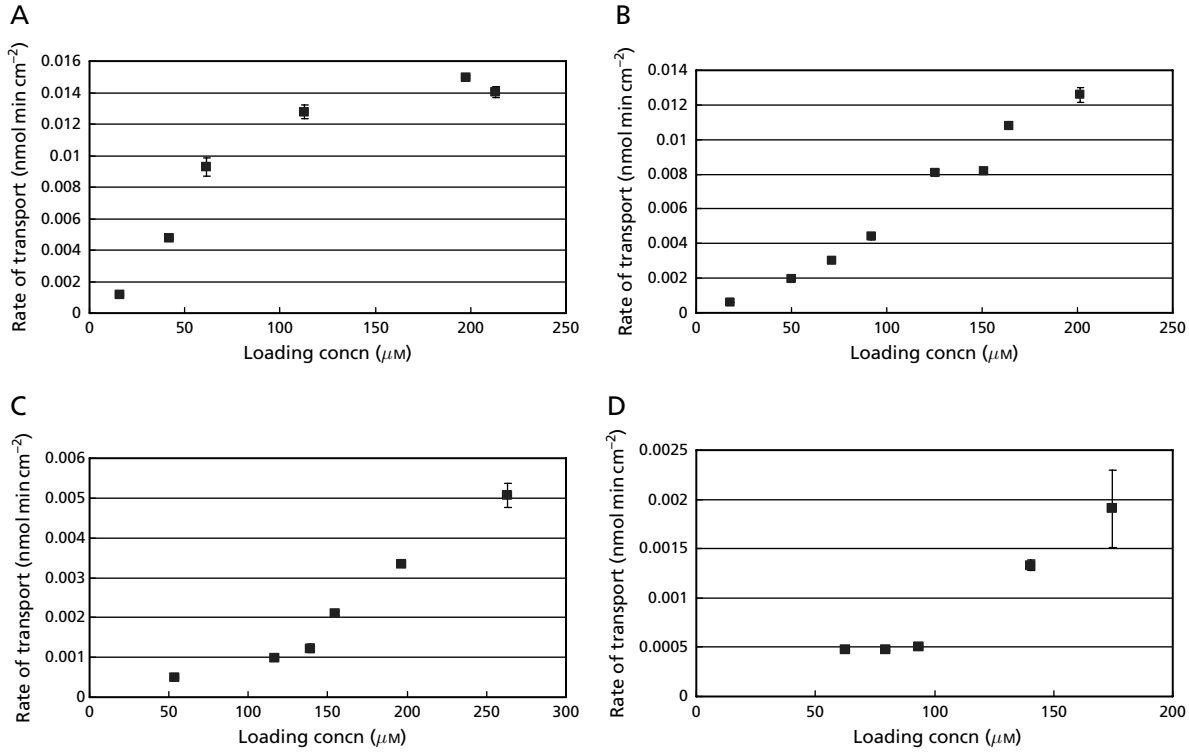

Figure 2 Basal-to-apical transport rate of epicatechin (A), epigallocatechin (B), epicatechin gallate (C) and epigallocatechin gallate (D) at different loading concentrations. Each point and vertical bar represented the mean ± s.e. of at least three determinations.

Due to the lack of authentic standards of the related metabolites for each GTC, it would have been difficult to quantify each of the metabolites. Although hydrolysis by glucuronidase/aryl sulfatase could provide a total concentration of parent compound plus the glucuronides and sulfate, such treatment would not be applicable to the quantification of the methylation metabolites of GTC identified in our previous study (Zhang et al 2004). Therefore, only parent compounds have been quantified and the efflux transport kinetics of the parent compounds were investigated in the present study.

P_{app} values of the efflux transport of the four major GTCs at concentrations close to 50 μM were significantly different from each other and followed the descending order: EC > EGC > ECG ≈ EGCG. Although P_{app} values (basolateral to apical) of the four GTCs at 50 μM obtained in the present study were slightly lower than those obtained in our previous study (Zhang et al 2004), the order of the extent of efflux transport was the same. Different passages of Caco-2 cells used may account for the differences in P_{app} values obtained in the two studies.

The profile of the rate of efflux transport versus concentration of EC showed that EC exhibited a carrier-mediated transport; the rate of transport increased as the EC concentration increased and approached a limited value at higher concentrations. The kinetic parameters obtained provide information for an overall saturability (K_m), capacity (V_{max}) and intrinsic clearance (V_{max}/K_m) of EC in the Caco-2 cell monolayer model in which more than one transporter may be involved. The kinetic parameters of EC ($K_m = 131\ \mu$M; $V_{max} = 0.0249$ nmol min cm^{-2}; $V_{max}/K_m = 0.19\ \mu$L min cm^{-2}) appeared to be comparable with those of other putative efflux substrates such as quercetin 4′-β-glucoside, a dietary flavonoid (K_m=43.6 μM; $V_{max} = 0.009$ nmol min cm^{-2}; $V_{max}/K_m = 0.21\ \mu$L min cm^{-2}), obtained in the Caco-2 cell monolayer model (Walgren et al 2000). The apparent affinity (K_m) of EC appeared to lie far above its plasma concentration (approx. 0.2 μM) obtained after oral administration of green tea to humans (Yang et al 1998). Hence, saturation of efflux transporters in the intestine may not occur with normal green tea consumption and efflux transporters would continue to play a role in reducing the oral bioavailability of EC.

For EGC, ECG and EGCG, the rate of efflux transport increased in a concentration-dependent manner and did not reach a plateau over the tested concentration range. According to the literature, EGC, ECG and EGCG should undergo carrier-mediated transport (Vaidyanathan & Walle 2001, 2003; Hong et al 2003; Zhang et al 2004). The failure to show a saturable process under the present experimental conditions may be due to low affinity for the efflux transporters. In order to saturate the transporters, a higher loading concentration of EGC, ECG and EGCG may be necessary. However, our experiments indicated that loading concentrations greater than 300 μM of EC and EGC would cause a significant drop in TEER values (below 480 Ω cm^2), suggesting that the integrity of the monolayers may have been damaged and thus limiting further investigation.

Intrinsic clearance of GTCs in the Caco-2 monolayer model followed the descending order of EC > EGC > ECG > EGCG, which may reflect the potential order of elimination occurring in the intestine. Moreover, the intrinsic clearance of EGC, ECG and EGCG appeared to be quite slow compared with that of EC. Therefore, the role of efflux transporters in limiting the oral bioavailability of EGC, ECG and EGCG may not be as significant as that of EC.

Conclusion

The extent of efflux transport of the four major GTCs in the Caco-2 monolayer model was EC > EGC > ECG ~ EGCG, which may reflect the order of elimination occurring in the intestine. Kinetic studies indicated that the saturable efflux transport of EC took place in the Caco-2 cell monolayer model. The kinetic parameters that give an indication of saturability ($K_m = 131\ \mu$M), capacity ($V_{max} = 0.0249$ nmol min cm^2) and intrinsic clearance ($V_{max}/K_m = 0.19\ \mu$L min cm^{-2}) of Caco-2 cells for EC provide information on the importance of efflux in reducing the oral bioavailability of EC. No saturation was observed for the efflux transport of EGC, ECG and EGCG, which may due to their weak affinity for the transporters.

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