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Transepithelial Transport of Theasinensins through Caco-2 Cell Monolayers and Their Absorption in Sprague–Dawley Rats after Oral Administration

Ju Qiu,[†] Yuko Kitamura,[†] Yuji Miyata,[‡] Shizuka Tamaru,[‡] Kazunari Tanaka,[‡] Takashi Tanaka,[§] and Toshiro Matsui*[,†]

[†]Department of Bioscience and Biotechnology, Division of Bioresource and Bioenvironmental Sciences, Faculty of Agriculture, Graduated School of Kyushu University, 6-10-1 Hakozaki, Fukuoka 812-8581, Japan

[‡]Department of Nutrition, University of Nagasaki, 1-1-1 Manabino, Nagayo-cho, Nishisonogi-gun, Nagasaki 851-2195, Japan

[§]Graduate School of Biochemical Science, Nagasaki University, 1-14 Bukyo-machi, Nagasaki 852-8521, Japan

Supporting Information

ABSTRACT: The aim of this study is to illustrate the *in vivo* and *in vitro* absorption of theasinensins B and A that are (−)-epigallocatechin-3-*O*-gallate (EGCG)−(−)-epigallocatechin (EGC) dimer and EGCG dimer, respectively, and their transport pathway across the intestinal membrane. Our animal study by a single oral administration to rats demonstrated the intact absorption of theasinensins into the blood system, which was estimated to be a >10-fold lower absorption amount than EGCG. The *in vitro* absorption study indicated that theasinensins can be transported across Caco-2 cell monolayers, while their permeability coefficients were also >10-fold lower than those of EGCG and EGC. Transport experiments using cytochalasin D or quercetin as a tight junction (TJ) modulator and a non-saturable permeation revealed that theasinensins were transported across Caco-2 cells in a TJ paracellular diffusion route. In conclusion, the dimers of condensed catechins, theasinensins B and A, can be absorbed intact into rat blood and transported across Caco-2 cell monolayers probably through a TJ paracellular pathway.

KEYWORDS: *Theasinensin, Caco-2 cell, tight junction, absorption, bioavailability*

INTRODUCTION

Tea, one of most popular beverages worldwide, is receiving increasing interest from scientists and consumers because of its health benefits for a variety of disorders. The beneficial effects of tea are attributed to its polyphenols, particularly catechins.[1] In green tea, enzymes catalyzing oxidation of catechins are inactivated by steaming or roasting immediately after crop, while in black tea, catechins are easily oxidized by the enzymes during the tea fermentation process to form condensed catechins.[2] Because (−)-epigallocatechin-3-*O*-gallate (EGCG) and (−)-epigallocatechin (EGC) account for over 70% of total tea catechins in tea leaves, their dimers, theasinensin B (an EGCG−EGC dimer) and theasinensin A (an EGCG dimer) (Figure 1) are one of the major oxidation products of condensed catechins in fermented tea.[3,4]

Theasinensins have been reported to show many biological properties in fermented tea. Our previous studies demonstrated that theasinensins were closely associated with the antihyperglycemic effect of fermented tea obtained from the mixture of green tea and loquat leaves.[5] Furthermore, theasinensins in oolong tea showed anticancer and anti-inflammatory effects.[6,7] Recent studies reported that theasinensins had an antiviral effect, similar to EGCG, on herpes simplex virus (HSV) infectivity.[8]

It should be noted that biological properties of polyphenols depend upon their bioavailability.[9] It has been reported that EGCG could be absorbed as an intact form in animals[10,11] and humans[12,13] after ingestion of EGCG or tea, while some catechins were metabolized or derivatized during the absorption process.[14] The Caco-2 cell monolayers derived from human colon carcinoma resemble morphologically the enterocytes of the small intestine and exhibit brush-border characteristics at the apical side after confluence. It has been widely used as an intestinal absorption model for studying

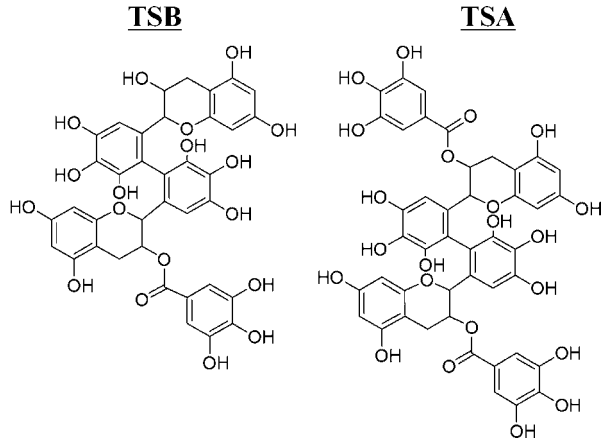

Figure 1. Chemical structures of theasinensins B and A.

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permeability and transport characteristics of drugs.[15] The relevant studies on the permeability of EGCG across Caco-2 cell monolayers illustrated that EGCG was slowly transported via the paracellular pathway.[16] Other catechins, such as EGC, were also reported to be transported across Caco-2 cell monolayers through active transporter, monocarboxylic acid transporter (MCT).[16,17]

In contrast to absorption studies on monomeric catechins, less study on the absorption of condensed catechins has been performed thus far, except procyanidins. It has been reported that procyanidins formed from catechin and epicatechin transport across Caco-2 cell monlayers via paracellular diffusion[18] and are also reported to be absorbed and detected efficiently as an intact form in rat plasma.[19] Thus, in this study, we investigate whether dimeric catechins, theasinensins, are absorbable compounds in *in vivo* rat experiments, together with *in vitro* experiments on their absorption characteristics in Caco-2 cells.

MATERIALS AND METHODS

Materials. Theasinensins B (TSB) and A (TSA) were prepared according to our previous methods.[2] Catechins (EGCG and EGC), benzoic acid, and phloretin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Grand Island, NY). Fluorescein (sodium salt) was from Sigma-Aldrich (St. Louis, MO). Quercetin was from Trade (TCI) Mark (Tokyo, Japan). Cytochalasin D was from Enzo Life Sciences (Farmingdale, NY). Other reagents were of analytical grade and used without further purification.

Animal Experiments. Experimental animals, nine male Sprague–Dawley rats (SPF/VAF Crj: SD, 7 weeks old, 290.6 ± 8.3 g) supplied by Charles River Japan (Kanagawa, Japan) were divided into three groups (TSB, TSA, and EGCG groups) (three rats in each group). The rats were acclimatized under laboratory conditions (21 ± 1 °C, 55.5 ± 5% humidity, and 12 h dark/light cycle) for 1 week before experiments, fed on an MF diet (Oriental Yeast, Tokyo, Japan), and given water *ad libitum*. The rats were fasted for 16 h prior to a single oral administration of theasinensins or EGCG at each dose of 100 mg/kg in 2 mL of saline solution. Before and after administration at a fixed time of 0.25, 0.5, 1, 2, 4, and 6 h, blood was taken from the tail vein to a heparinized tube and then centrifuged at 3500*g* for 15 min at 4 °C to obtain the plasma sample. All rats were handled in accordance with the Guidance for Animal Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (number 105, 1973) and Notification (number 6, 1980 of the Prime Minister's Office) of the Japanese Government.

Determination of Theasinensins in Blood. An aliquot (100 μL) of plasma was deproteinized by 100 μL of 4% trichloroacetic acid and centrifuged at 10000*g* for 20 min at 4 °C. The supernatant was evaporated to dryness and then dissolved in 100 μL of 0.1% trifluoroacetic acid, followed by centrifugation at 10000*g* for 20 min at 4 °C. The supernatant was collected and applied to a Sep-Pak Plus C_{18} cartridge (Waters, Milford, MA) with an elution of 3.0 mL of 10 or 40% acetonitrile containing 0.1% trifluoroacetic acid for TSB and TSA, respectively. The eluate was then evaporated to dryness and dissolved in 10% acetonitrile containing 50 mM phosphate (pH 6.8) for further analysis via electrochemical detector–high-performance liquid chromatography (ECD–HPLC).

Determination of Theasinensins by ECD–HPLC. TSB and TSA were determined by HPLC equipped with an ECD (Coulochem III, ESA, Bedford, MA). The column used for the system was a Cosmosil $5C_{18}$-MS II (4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan), and isocratic elution was performed by 10% acetonitrile containing 50 mM phosphate (pH 6.8) at 0.4 mL/min at 37 °C. The potentials of the ECD were set at 100 mV for the reference electrode and 500 mV for the working electrode. The dried sample dissolved in 20 μL of 10% acetonitrile containing 50 mM phosphate (pH 6.8) was injected into the ECD–HPLC system.

Pharmacokinetic Analysis of Theasinensins in Plasma. The pharmacokinetic parameters, such as C_{max} and T_{max}, were analyzed from 0 to 6 h after administration to SD rats. The half-life ($t_{1/2}$) was calculated by plotting the logarithmic plasma level against time. The $AUC_{0-6\ h}$ was calculated using the trapezoidal rule. All pharmacokinetic analyses were performed with a GraphPad Prism 5 Software (La Jolla, CA).

Cell Culture. Caco-2 cells were cultured in DMEM supplemented with 10% FBS, 1% non-essential amino acids, 2 M L-glutamine, 100 units/mL penicillin, 100 mg/mL streptomycin, and 1.7 mM insulin. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The monolayers became confluent 4–5 days after seeding at a number of 1×10^6 cells per 100 mm dish (Nitta Gelatin, Osaka, Japan), and the cells were passaged at a split ratio of 4–8 by trypsinization with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS). Caco-2 cells used in this study were between passages 50 and 60.

For transport experiments, Caco-2 cells were seeded at a density of 4×10^4 cells/mL on transwell inserts (polycarbonate membrane, 0.4 μm pore size, 12 mm, Costar, Bodenheim, Germany) coated with type I collagen (collagen gel culturing kit, Cellmatrix, Nitta Gelatin). The cells were cultured using the BD BioCoat intestinal epithelium differentiation kit (BD Biosciences, Bedford, MA), and the medium was changed everyday. Monolayers were formed after culturing for 5 days. The integrity of the cell monolayers was evaluated by measuring the transepithelial electrical resistance (TEER) with a multichannel voltage/current ECV-4000 system (World Precision Instruments, Sarasota, FL). Monolayers with TEER of 200–400 Ω cm^2 were used for the experiments. The lower TEER values of ca. 100 Ω cm^2 were prepared by treating Caco-2 cells with cytochalasin D (10 μg/mL) for 20 min before adding test compounds in the process of transport experiments, because cytochasasin D as a cell-permeable mycotoxin was reported to decrease the intestinal barrier function by disrupting actin filaments and inhibiting actin polymerization.[20] Otherwise, the higher TEER values of ca. 400 Ω cm^2 were prepared by treating cells with 200 μM quercetin for 24 h before transport experiments, because quercetin was reported to enhance intestinal barrier function through the assembly of zonnula occludens-2, occludin, and claudin-1 and the expression of claudin-4 in Caco-2 cells.[21,22]

Theasinensin Transport. A transport experiment was conducted as described previously,[23] with minor modifications. Caco-2 cell monolayers grown in the transwell inserts were rinsed with Hank's balanced salt solution (HBSS) buffer (16 g/L NaCl, 0.8 g/L KCl, 2 g/L D-glucose, 0.7 g/L $NaHCO_3$, 0.24 g/L $NaHPO_4 \cdot 12H_2O$, 0.12 g/L KH_2PO_4, 0.4 g/L $MgSO_4 \cdot 7H_2O$, and 0.37 g/L $CaCl_2 \cdot 2H_2O$) and mounted in a Ussing Chamber system (model U-2500, Warner Instrument Corporation, Hamden, CT). An aliquot (6.0 mL) of HBSS buffer containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6.0) was added to the apical side, and HBSS buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4) was added to the basolateral side. After equilibration for 15 min at 37 °C, theasinensins (200 μM–2.0 mM) dissolved in apical HBSS buffer in the presence or absence of inhibitors (10 mM benzoic acid and 300 μM phloretin) were added to the apical side, and the basolateral side was replaced with fresh buffer. During the experiments, solutions in both sides were continuously bubbled with a mixture of O_2/CO_2 (95:5). After a 60 min transport experiment, 5.0 mL of basolateral solution was subjected to a Sep-Pak Plus C_{18} cartridge with an elution of 40% acetonitrile containing 0.1% trifluoroacetic acid, following the evaporation to dryness under vacuum. The dried sample was further analyzed by an ECD–HPLC system, as mentioned above.

The permeability coefficient (P_{app}) expressed in cm/s was determined as

$$P_{app} = dC/dtV/(AC_0)$$

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where dC/dt is the change in concentration on the receiving side over time (mmol/s), V is the volume of the solution in the receiving compartment (6.0 mL), A is the surface area of the membrane (0.2826 cm^2), and C_0 is the initial concentration in the donor chamber (mmol). The effect of transport inhibitors on P_{app} was expressed relative to the control (%).

Fluorescein Transport. Transport experiments of fluorescein were conducted with the Ussing Chamber system using Caco-2 cell monolayers mentioned above. Fluorescein (200 μM) in the presence or absence of 500 μM theasinensins (TSB and TSA) was added to the apical side (pH 6.0). During a 30 min incubation time, 100 μL of transported solution was taken from basolateral side per 5 min and replaced with an equal volume of fresh HBSS buffer (pH 7.4). The Lineweaver–Burk plots assayed the fluorescein transport from 10 to 200 μM in the presence or absence of 200 and 500 μM TSB. The fluorescence intensity of collected solution was measured with a fluorescence spectrophotometer (Wallac ARVO SX 1420 multilabel counter, Perkin-Elmer Life Sciences, Tokyo, Japan) at an excitation wavelength of 490 nm and an emission wavelength of 535 nm.

Statistical Analysis. Results are expressed as the mean ± standard error of the mean (SEM). Statistical differences within a group were evaluated by one-factor analysis of variance (ANOVA), followed by Tukey–Kramer's t test for *post-hoc* analysis. Other statistical evaluations were performed by Student's t test. Analyses were performed with a Stat View J 5.0 (SAS Institute, Cary, NC).

RESULTS

Absorption of Theasinensins into Blood of SD Rats. Figure 2 shows typical ECD–HPLC chromatograms of TSB (Figure 2a) and TSA (Figure 2b) in plasma from SD rats before and after administration. Under the present ECD–HPLC conditions, TSB and TSA standards were detected with high sensitivities of 10 and 50 nM, respectively (panel 1 of Figure 2). In addition, the ECD–HPLC system showed a good reproducibility with <10% coefficient of variation (n = 6), as well as R^2 values of calibration curves with >0.993. In comparison to the chromatograms of the standards, two peaks corresponding to TSB and TSA were detected at 16.9 and 36.7 min, respectively, in SD rat plasma at 1 h after administration (100 mg/kg) (panel 3 of Figure 2), while no corresponding peaks were detected in blank plasma (panel 2 of Figure 2). It indicates that theasinensins were absorbed intact *in vivo*.

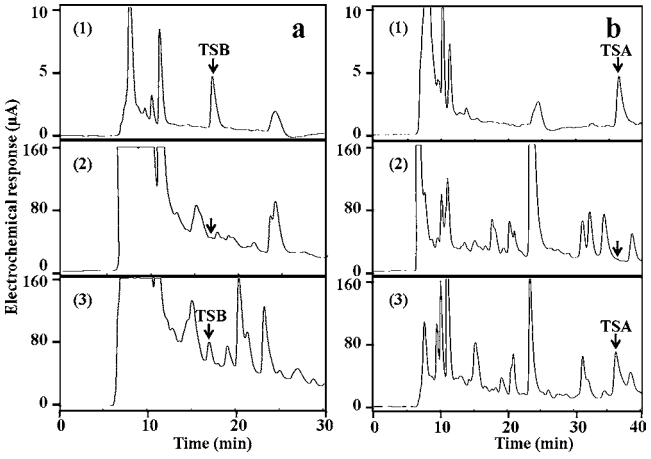

Figure 2. Typical ECD–HPLC chromatograms of plasma from SD rats after administration of (a) TSB or (b) TSA: (1) standard (25 nM TSB and 100 nM TSA), (2) plasma before administration, and (3) plasma at 1 h after administration of theasinensins (100 mg/kg). HPLC and ECD conditions are described in the Materials and Methods.

The pharmacokinetic profiles of theasinensins and EGCG are illustrated in Figure 3, and their pharmacokinetic parameters are summarized in Table 1. TSB (C_{max}, 7.5 ± 1.0 nM; $AUC_{0-6\ h}$, 21.5 ± 0.7 nmol h L^{-1}) and TSA (C_{max}, 9.3 ± 1.8 nM; $AUC_{0-6\ h}$, 16.7 ± 1.0 nmol h L^{-1}) reached the maximum absorption at 1 h, and there was no significant difference in their C_{max} and AUC values. However, both C_{max} and AUC values of theasinensins were much lower than that of EGCG (C_{max}, 110.5 ± 35.4 nM; $AUC_{0-6\ h}$, 275.4 ± 45.3 nmol h L^{-1}).

Permeability of Theasinensins across Caco-2 Cell Monolayers. TSB (Figure 4a) and TSA (Figure 4b) in the transported (or basolateral) solution after the 60 min transport experiments of 200 μM theasinensins were successfully detected by the ECD–HPLC system, indicating that both TSB and TSA can transport as an intact form across Caco-2 cell monolayers. The permeation of theasinensins and catechins under a proton gradient (pH 6.0 at the apical side and pH 7.4 at the basolateral side) was evaluated by the permeability coefficient (P_{app}) (Table 2). The P_{app} value of TSB ($9.7 \pm 1.3 \times 10^{-9}$ cm/s) was not significantly different from that of TSA ($1.5 \pm 0.3 \times 10^{-8}$ cm/s) but much lower than those of catechins (EGCG, $1.7 \pm 0.4 \times 10^{-7}$ cm/s; EGC, $2.0 \pm 0.1 \times 10^{-7}$ cm/s).

Theasinensin Transport under a Proton Gradient. As shown in Figure 5a, TSB and TSA at a concentration of 500 μM significantly inhibited the fluorescein transport across Caco-2 cell monolayers ($p < 0.01$) under a proton gradient. The inhibitions of TSB and TSA against fluorescein transport were 40.3 ± 6.7 and 47.0 ± 7.6% of the control, respectively, and they were not significantly different. The Lineweaver–Burk plot (Figure 5b) clearly shows that the inhibition mode of TSB toward fluorescein transport across Caco-2 cell monolayers was noncompetitive, as well as TSA (data not shown). Further experiments using 10 mM benzoic acid, a MCT substrate, and 300 μM phloretin, a MCT inhibitor, were performed for transport experiments of 500 μM TSB and TSA. As a result, neither benzoic acid nor phloretin significantly affected the transport of TSB (Figure 5c) or TSA (Figure 5d). Our preliminary studies of theasinensins showed that their P_{app} values under no proton gradient (TSB, $8.1 \pm 1.9 \times 10^{-9}$ cm/s; TSA, $9.3 \pm 3.7 \times 10^{-9}$ cm/s, at pH of 7.4 in both sides) were similar to those under a proton gradient (Table 2). These results and no significant change of P_{app} of theasinensins in the bidirectional transport study (data not shown) suggested that TSB and TSA did not transport via MCT across Caco-2 cell monolayers, although theasinensins might act as a MCT inhibitor, so that MCT-mediated transport of fluorescein was inhibited by theasinensins, as shown in panels a and b of Figure 5.

Paracellular Transport of Theasinensins. Figure 6 shows the relationship between the permeable velocity of theasinensins and their concentration for 60 min. The permeation rate of TSB (Figure 6a) and TSA (Figure 6b) increased with the concentration without any saturation up to 2.0 mM. Possible paracellular transport of theasinensins was then characterized using Caco-2 cell monolayers with different TEER values (Figure 7). The P_{app} values of theasinensins across Caco-2 cells treated with cytochalasin D lowering the TEER (panels a and b of Figure 7) were significantly higher than those across untreated cells ($p < 0.01$). On the other hand, quercetin increasing the TEER, namely, enhancing intestinal barrier function (panels c and d of Figure 7), caused the significant

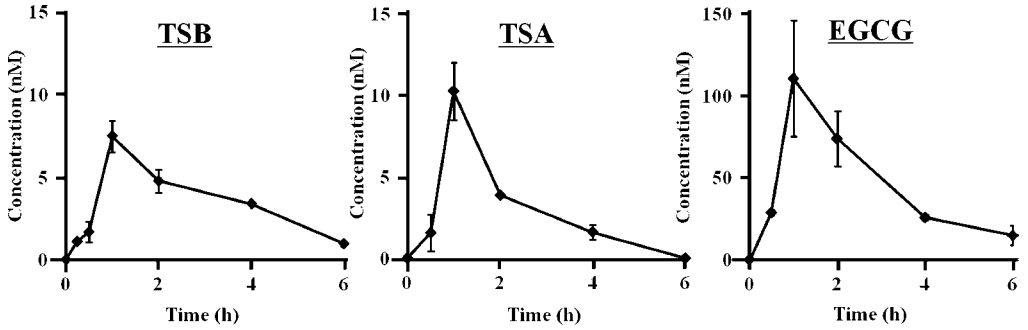

Figure 3. Time course of plasma concentrations of TSB, TSA, and EGCG in SD rats after a single oral administration of 100 mg/kg for each compound. Results are expressed as the mean ± SEM (n = 3).

Table 1. Pharmacokinetic Parameters of Theasinensins and EGCG after a Single Oral Administration to SD Rats[a]

	TSB	TSA	EGCG
C_{max} (nM)	7.5 ± 1.0 a	9.3 ± 1.8 a	110.5 ± 35.4 b
T_{max} (h)	1.0	1.0	1.0
$AUC_{0-6\ h}$ (nmol h L^{-1})	21.5 ± 0.7 a	16.7 ± 1.0 a	275.4 ± 45.3 b
$t_{1/2}$ (h)	1.8	1.2	1.7

[a]Results are expressed as the mean ± SEM (n = 3). Statistical differences among each group were evaluated by Tukey–Kramer's t test. Values with different letters significantly differed at $p < 0.01$. T_{max}, time to reach maximum concentration; C_{max}, maximum concentration; $t_{1/2}$, half-life; AUC, area under the curve. T_{max} and $t_{1/2}$ were calculated by plotting test compound levels at 1, 2, 4, and 6 h against the logarithmic concentration. C_{max} was defined as the maximum concentration of the compound among the levels obtained at each fixed time.

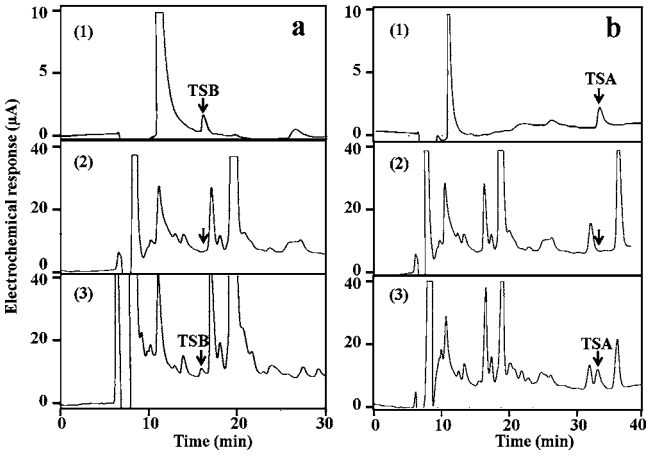

Figure 4. Typical ECD–HPLC chromatograms of (a) TSB and (b) TSA in Caco-2 cells transport experiments: (1) standard (10 nM TSB and 50 nM TSA), (2) transported solution in the absence of theasinensins, and (3) transported solution in the presence of 200 μM theasinensins. HPLC and ECD conditions are described in the Materials and Methods.

decrease of P_{app} values of theasinensins ($p < 0.01$). It means that the transport of TSB (panels a and c of Figure 7) or TSA (panels b and d of Figure 7) was inversely correlated with the TEER values, indicating that theasinensins may transport across Caco-2 cells via paracellular pathways. This finding also suggests that the intestinal absorption of TSB and TSA may be restricted when the epithelial tight junction (TJ) is tight enough.

Table 2. Permeability Coefficient (P_{app}) of Theasinensins and Catechins Across Caco-2 Cell Monolayers[a]

compounds	P_{app} (cm/s)
TSB	$9.7 \pm 1.3 \times 10^{-9}$ a
TSA	$1.5 \pm 0.3 \times 10^{-8}$ a
EGCG	$1.7 \pm 0.4 \times 10^{-7}$ b
EGC	$2.0 \pm 0.1 \times 10^{-7}$ b

[a]P_{app} values were detected under a proton gradient (pH 6.0 at the apical side and pH 7.4 at the basolateral side). Results are expressed as the mean ± SEM (n = 5). Statistical differences among groups were evaluated by Tukey–Kramer's t test. Values with different letters significantly differed at $p < 0.05$.

DISCUSSION

Theasinensins have been reported as the major oxidative products of catechins in fermented tea,[3,24] and many researchers have paid attention to their synthesis[2,25] and bioactivities for health benefits[5−8] recently. However, although intact absorption of catechins into the blood system has been reported thus far,[10−13] few studies on the absorption of condensed catechins were conducted.[19]

Quantification of theasinensins was successfully performed by an ECD–HPLC system that provided sufficient sensitivity as high as 10 nM for TSB and 50 nM for TSA without significant matrix interferences (Figures 2 and 4). Electrospray ionization–time-of-flight–mass spectrometry (ESI–TOF–MS) failed to reach such a high sensitivity (data not shown), because the ionization efficiency of theasinensins was too poor in the negative mode. The bioavailability of theasinensins was illustrated by the oral administration of theasinensins (100 mg/kg) to SD rats, and we found their intact absorption in rat plasma for the first time (Figure 2). The pharmacokinetic analysis revealed that theasinensins were rapidly absorbed intact to the rat blood system with short T_{max} of 1 h, which was shorter than caffeic or ferulic acid (2 h).[26] The C_{max} and $AUC_{0-6\ h}$ values of TSB (C_{max}, 7.5 ± 1.0 nM; $AUC_{0-6\ h}$, 21.5 ± 0.7 nmol h L^{-1}) and TSA (C_{max}, 9.3 ± 1.8 nM; $AUC_{0-6\ h}$, 16.7 ± 1.0 nmol h L^{-1}) were about 10 times lower than that of EGCG (C_{max}, 110.5 ± 35.4 nM; $AUC_{0-6\ h}$, 275.4 ± 45.3 nmol h L^{-1}). The C_{max} values of theasinensins were also much lower than those of caffeic acid (1 μM; 126 mg/kg administration) and ferulic acid (1.5 μM; 136 mg/kg administration),[26] suggesting the lower absorption of theasinensins in rats. As some researchers reported, catechins were in part absorbed intact but extensively converted to glucuronide and/or sulfate conjugates during their transport through Caco-2 cell

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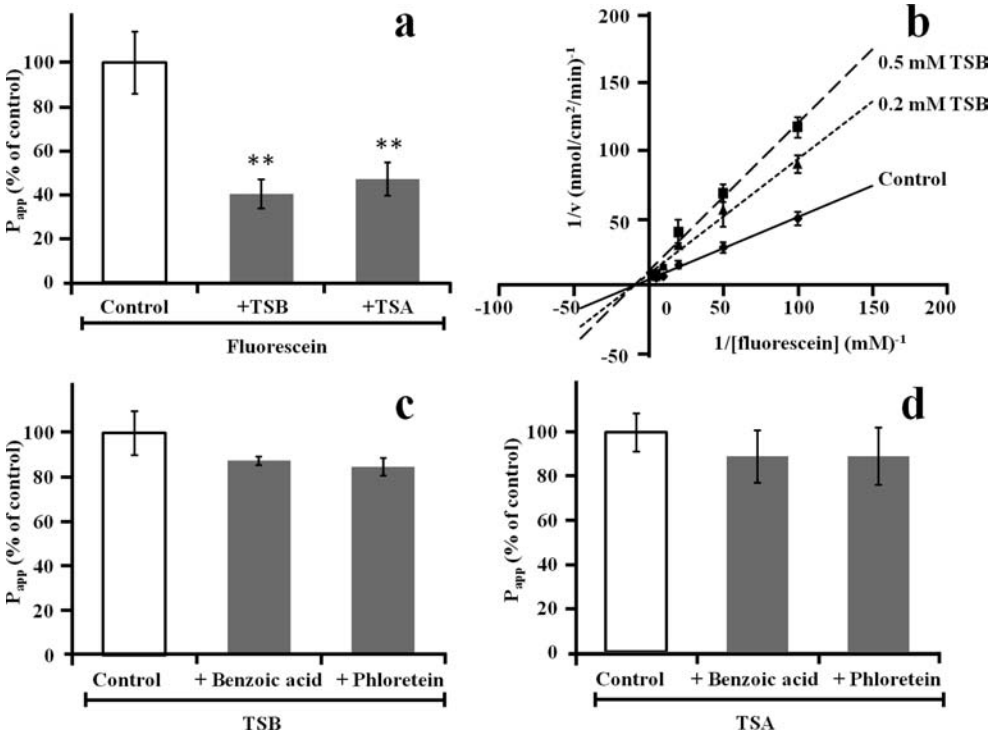

Figure 5. Theasinensin transport across Caco-2 cell monolayers from the apical side at pH 6.0 to the basolateral side at pH 7.4. (a) Effect of TSB and TSA (each 500 μM) on the permeability coefficient (P_{app}) of 200 μM fluorescein. (b) Lineweaver–Burk plots of fluorescein (10–200 μM) transport in the presence or absence of TSB (200 and 500 μM). (c) Permeability of TSB (500 μM) in the presence or absence of benzoic acid (10 mM) and phloretin (300 μM). (d) Permeability of TSA (500 μM) in the presence or absence of benzoic acid (10 mM) and phloretin (300 μM). Results are expressed as the mean ± SEM (n = 3). (**) $p < 0.01$ versus the control by Student's t test.

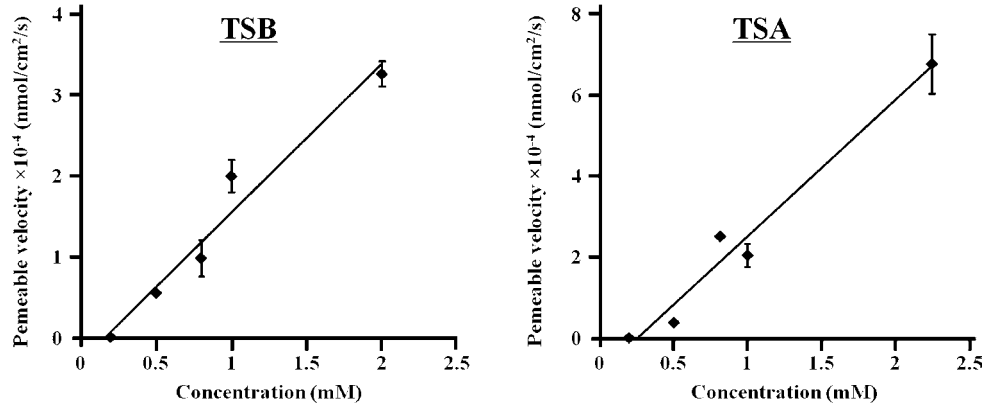

Figure 6. Concentration dependence of TSB and TSA (each 200 μM–2 mM) transports across Caco-2 cell monolayers from the apical side at pH 6.0 to the basolateral side at pH 7.4. Results are expressed as the mean ± SEM (n = 3).

monolayers.[27,28] The possibility of any metabolism of theasinensins for the explanation of their low absorption cannot be excluded, because Neilson et al.[29] have pointed out the methylation or sulfation of theasinensins in Caco-2 cells. In this study, the concentration of theasinensins in the apical side of Caco-2 cells before and after 60 min transport experiments was observed (data not shown), but there was no significant change, indicating that no degradation of theasinensins happened in the apical side of Caco-2 cells. However, a further study must be required to clarify whether theasinensins are metabolized to some conjugates during *in vivo* absorption, as demonstrated in our previous metabolism study of polyphenols in rat blood.[30]

However, it is noteworthy that theasinensins could be absorbed intact in SD rat plasma. The next studies focused on the intact absorption mechanism of theasinensins by the Caco-2 cell model. The *in vitro* transepithelial absorption of theasinensins across Caco-2 cell monolayers was significantly lower than that of catechins ($p < 0.05$) because the P_{app} values of EGCG ($1.7 \pm 0.4 \times 10^{-7}$ cm/s) and EGC ($2.0 \pm 0.1 \times 10^{-7}$ cm/s) were over 10-fold higher than those of theasinensins (TSB, $9.7 \pm 1.3 \times 10^{-9}$ cm/s; TSA, $1.5 \pm 0.3 \times 10^{-8}$ cm/s) (Table 2), which was parallel to the prediction from *in vivo* absorption. The lower permeability of condensed catechins (or theasinensins) compared to monomers was in agreement with the report by Deprez et al.,[18] who revealed over 10-fold lower

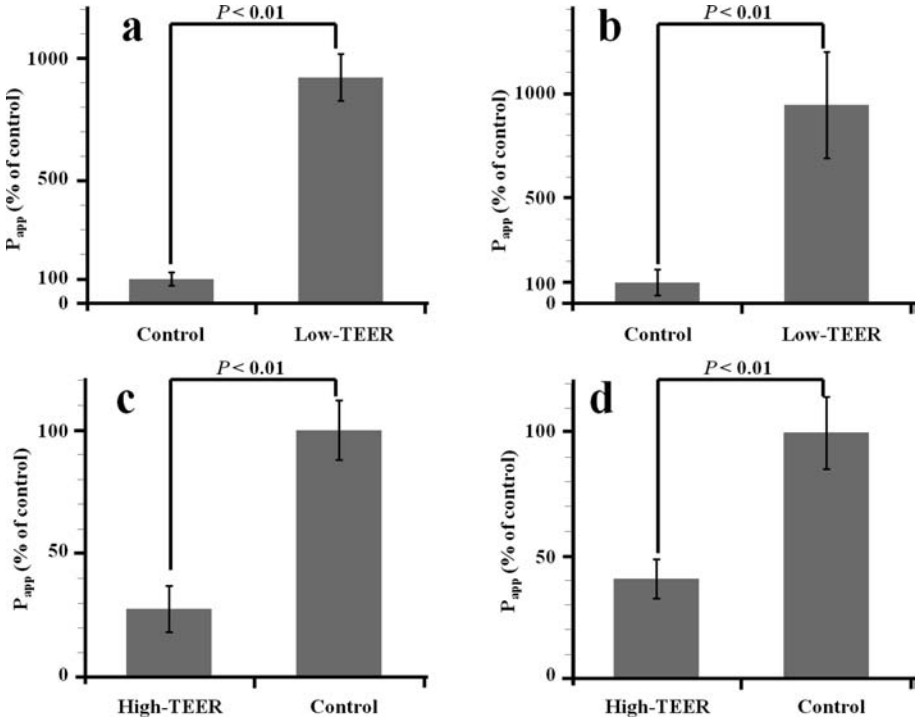

Figure 7. Correlation between TEER of Caco-2 cell monolayers and P_{app} values of (a and c) TSB and (b and d) TSA. Theasinensins (500 μM) were loaded in the apical side. Different TEER values were prepared by 10 μg/mL cytochalasin D (a and b, low TEER) or 200 μM quercetin (c and d, high TEER). TEER values (Ω cm^2) are indicated as follows: (a) control (normal cell without treatment), 376.1 ± 24.4; low TEER, 105.6 ± 4.9; (b) control, 252.7 ± 16.7; low TEER, 92.5 ± 15.5; (c) high TEER, 423.1 ± 4.6; control, 225.6 ± 11.1; and (d) high TEER, 382.7 ± 10.6; control, 202.5 ± 25.5. Results are expressed as the mean ± SEM (n = 3). p < 0.01 versus the control group by Student's t test.

transport of polymeric procyanidins than that of monomeric catechins.

There were conflicting results regarding the transport mechanism of catechins. Some researchers have reported that the accumulation of gallated catechins in the epithelial Caco-2 cells was mediated by the proton transporter MCT,[17] but others suggested that catechins were slowly transported via the paracellular pathway.[16] Thus, in this study, first of all, we examined the involvement of MCT in the transport of theasinensins using fluorescein, a fluorescent marker dye that was transported via MCT under a proton gradient (pH 6.0 at the apical side and pH 7.4 at the basolateral side).[31] As a result, TSB and TSA inhibited the fluorescein transport in a noncompetitive manner (panels a and b of Figure 5). Together with the subsequent results that neither TSB nor TSA transport was affected by benzoic acid or phloretin (panels c and d of Figure 5), it was concluded that theasinensins were not transported by MCT. This finding was in agreement with the studies by Konishi et al.[16] arguing that catechins, such as EC, EGC, and EGCG, were unlikely transported by MCT as substrates even though they inhibited fluorescein transport under a proton gradient.[16,32] However, the noncompetitive inhibition of theasinensins on flurescein transport provided an alternative possibility that they may affect MCT expression or translocation, such as quercetin and phloretin.[33,34]

Our further experiments of theasinensin transport under a proton gradient revealed that the transport of TSB and TSA increased linearly and did not show a plateau up to 2.0 mM under our experimental conditions (Figure 6). This is consistent with the character of Lucifer Yellow, a marker for paracellular transport, which also showed non-saturable absorption in Caco-2 cells.[31] Konishi et al.[32] have pointed out that saturable transport under a proton gradient is one of characteristics of carrier-mediated transport, while concentration-dependent and non-saturable transport supports the paracellular pathway via TJ. Moreover, the results of the correlation between TEER of Caco-2 cell monolayers and P_{app} values of theasinensins (Figure 7) supported the key role of TJ in theasinensin transport, similar to the reported paracellular diffusion of large polyphenols, such as hesperidin[20] and procyanidins.[18]

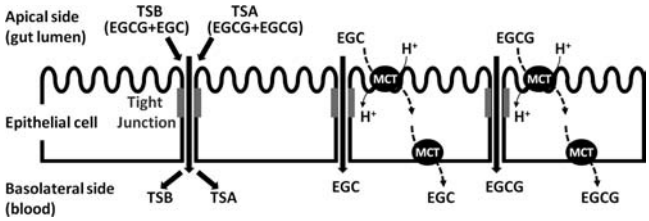

Figure 8. Possible routes for absorption of theasinensins and catechins. MCT = monocarboxylic acid transporter.

The intestine is the first potential tissue responsible for the first-pass metabolism of polyphenols after oral absorption. Given the present results of the *in vitro* transport pathway, the possible routes for absorption of theasinensins and catechins were proposed (Figure 8). TSB (dimer of EGCG and EGC) and TSA (dimer of EGCG) may transport the intestinal membrane via TJ paracellular passive diffusion, while EGCG and EGC may mainly transport by paracellular pathways, although partially by MCT.

In conclusion, the present study demonstrated the first finding that dimeric catechins of TSB and TSA were intact absorbable natural compounds in the rat blood system, while their absorption (or permeability) was lower than their monomers. The transport of theasinensins across Caco-2 cell monolayers would be involved in the TJ paracellular pathway but not the MCT-mediated transport. Further studies regarding metabolism and accumulation of absorbed theasinensins and their conjugates are required to delineate the importance of *in vivo* mechanisms in animals or humans. On the other hand, owing to their reported biological activities, such as antihyperglycemia, anticancer, and anti-inflammation, the improvement of their poor absorption should be considered in the future.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Typical ECD−HPLC chromatograms of EGCG in SD rat experiments (Figure S1), typical ECD−HPLC chromatograms of EGCG and EGC in Caco-2 cells transport experiments (Figure S2), Lineweaver−Burk plots of fluorescein transport in the presence or absence of TSA (Figure S3), and PDA−HPLC chromatograms of theasinensins B and A on the apical side of Caco-2 cell monolayers (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +81-92-642-3011. Fax: +81-92-642-3011. E-mail: tmatsui@agr.kyushu-u.ac.jp.

Notes

The authors declare no competing financial interest.

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