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Transepithelial transport of flavanone in intestinal Caco-2 cell monolayers

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Abstract

Our recent study [S. Kobayashi, S. Tanabe, M. Sugiyama, Y. Konishi, Transepithelial transport of hesperetin and hesperidin in intestinal Caco-2 cell monolayers, Biochim. Biophys. Acta, 1778 (2008) 33–41] shows that the mechanism of absorption of hesperetin involves both proton-coupled active transport and transcellular passive diffusion. Here, as well as analyzing the cell permeability of hesperetin, we also study the transport of other flavanones, naringenin and eriodictyol, using Caco-2 cell monolayers. Similar to hesperetin mentioned, naringenin and eriodictyol showed proton-coupled polarized transport in apical-to-basolateral direction in non-saturable manner, constant permeation in the apical-to-basolateral direction ($J_{ap \rightarrow bl}$) irrespective of the transepithelial electrical resistance (TER), and preferable distribution into the basolateral side after apical loading in the presence of a proton gradient. Furthermore, the proton-coupled $J_{ap \rightarrow bl}$ of hesperetin, naringenin and eriodictyol, were inhibited by substrates of the monocarboxylic acid transporter (MCT), such as benzoic acid, but not by ferulic acid. In contrast, both benzoic and ferulic acids have no stimulatory effect on $J_{ap \rightarrow bl}$ of each flavanone by *trans*-stimulation analysis. These results indicates that proton-driven active transport is commonly participated in the absorption of flavanone in general, and that its transport is presumed to be unique other than MCT-mediated transport for absorption of phenolic acids (PAs), sodium-dependent MCT (SMCT) nor anion exchanger-mediated transport.

Keywords: Flavanone; Hesperetin; Naringenin; Eriodictyol; H^+-coupled active transport; Transcellular passive diffusion; Phenolic acid; MCT; Caco-2

Flavanones, such as hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone), naringenin (4′,5,7-trihydroxyflavanone), and eriodictyol (3′,4′,5,7-tetrahydroxyflavanone), show a limited distribution (i.e. specific to citrus fruits) compared to other flavonoids. Recently, citrus flavanones have received attention because of their potential therapeutic benefits [2,3].

Flavanones are commonly present as glycosides in intact plants. Several *in vivo* studies reported that flavanone glycosides are absorbed as their respective hydrolyzed aglycones [3–5]. Further, it was also reported that hesperetin and naringenin are both bioavailable, and naringenin seemingly more so than hesperetin in human subjects through the pharmacokinetic analysis of hesperetin and naringenin after intake of each aglycone or blood orange juice [5,6]. Flavonoids and isoflavonoids, such as quercetin, daidzein or genistein, are generally considered to be absorbed by passive diffusion because of their affinity for biomembranes [7,8]. Nevertheless, a thorough analysis of the absorption mechanisms of flavonoids and their glycosides has hitherto not been reported. Analysis of this kind can reveal some interesting results as demonstrated in the case of artepillin C, an active constituent of Brazilian propolis [9]. It might be the case that SGLT1 (sodium-dependent glucose cotransporter-1) and MRP2 (multidrug resistance-associated protein-2) are involved in absorption or efflux of quercetin 4′-β-glucoside across intestinal tract [10,11]. Thus the absorption and metabolism of flavonoids are complex.

We recently showed that in Caco-2 cells hesperetin is absorbed *via* proton-coupled active transport, in addition to the transcellular passive diffusion [1]. The H^+-driven $J_{ap \rightarrow bl}$ of hesperetin was inhibited by benzoic acid, but

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not by glycylsarcosine (Gly-Sar) or phloretin, a substrate of PEPT1 (H^+-coupled peptide transporter-1) and an inhibitor of MCT, respectively [1]. Nonetheless, the mechanism of H^+-driven polarized absorption is still unclear. In this study, we have examined and clarified the absorption mechanism of other citrus flavanones, such as naringenin and eriodictyol together with hesperetin, using Caco-2 cells as an *in vitro* model of intestinal absorption and metabolism [12].

Materials and methods

Materials. The human colon adenocarcinoma cell line, Caco-2, was obtained from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids, penicillin, streptomycin, gentamycin, and Hanks' balanced salt solution (HBSS) were all from Invitrogen Corp. (Carlsbad, CA, USA). Fetal calf serum (FCS) was obtained from ICN Biomedicals, Inc. (Osaka, Japan), and type-I collagen solution was from Nitta Gelatin (Osaka, Japan). Transwell cell culture chambers (pore size: 0.4 μm; diameter: 12 mm) and the Millicell-ERS volt-ohmmeter with Ag/AgCl electrodes were purchased from Coster (Cambridge, MA, USA) and from Nihon Millipore (Tokyo, Japan), respectively. Hesperetin, naringenin, and eriodictyol were from Wako Pure Chemicals Inc., Ltd. (Osaka, Japan), Sigma (St. Louis, MO, USA), and Extrasynthese (Basel, Switzerland), respectively. All other chemicals used in this study were of analytical grade.

Cell culture. Caco-2 cells were cultured in DMEM containing 10% FCS, 1% non-essential amino acids, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 μg/ml gentamycin in a humidified atmosphere of 5% CO_2 at 37 °C. Passages 60–90 were used.

Transepithelial transport experiments. Cells were seeded into Transwell inserts coated with type-I collagen. The cells were seeded at a density of 1×10^5 cells/cm^2 and a monolayer was formed after culturing for 2 weeks. The integrity of the cell layer was evaluated by measurement of transepithelial electrical resistance (TER) with Millicell-ERS equipment. The monolayer of cells was gently rinsed twice with HBSS and left to equilibrate in the same solution for 30 min at 37 °C. A monolayer with a TER of more than 300 Ω cm^2 was used for the transepithelial transport experiments. To obtain Caco-2 monolayers with various TER values, the monolayer cells were treated with cytochalasin D from the apical side. These monolayers were also used for permeation studies, as previously reported [13]. TER of the monolayer was measured before and after each transport experiment.

To measure the apical-to-basolateral permeability, 1.5 ml of HBSS (pH 7.4, 37 °C) was added to the basolateral chamber of the Transwell insert and then 0.5 ml of the test solution (pH 6.0 or 7.4, 37 °C) containing flavanone (50 μmol/l) was added to the apical side. Flavanones were dissolved in dimethyl sulfoxide (DMSO) and diluted with HBSS prior to starting the experiment. The resulting final concentration of DMSO, 1%, did not affect the transport [14]. After the desired incubation time at 37 °C, the basolateral solution was collected and then replaced with an equal volume of HBSS. The amount of flavanones transported by the Caco-2 cells was determined using an HPLC-electrochemical detector (ECD) with an ESA coulometric detection system (ESA Inc., Boston, MA, USA). The results were expressed in terms of specific permeability (μl/cm^2), which was calculated as the amount of compound transported divided by the initial concentration in the donor compartment. To examine the basolateral-to-apical transport, HBSS (pH 6.0 or 7.4, 37 °C) was added to the apical side and 1.5 ml of the test solution (pH 7.4, 37 °C) was added to the basolateral side.

For measurement of the countertransport effect, 10 mM ferulic acid and benzoic acid were preloaded into apical side for 20 min. Those compounds were then aspirated off and the cells were rapidly washed twice with HBSS before immediately adding the test solution to the apical side. Thereafter, the transport study was performed according to the method described earlier.

Chromatographic conditions. HPLC-ECD fitted with a coulometric detection system was used for analysis as previously reported [15,16]. Chromatographic separation was performed on a C18 column (ODS150, MC Medical, Inc., Tokyo, Japan). The mobile phase A (solvent A) was 50 mM sodium acetate containing 5% methanol (pH 3.0), while mobile phase B (solvent B) was 50 mM sodium acetate containing 40% acetonitrile and 20% methanol (pH 3.5). The elution profile (0.6 ml/min) was as follows: 0–0.5 min, isocratic elution, 60% solvent A/40% solvent B; 0.5–28.5 min, linear gradient from 60% solvent A/40% solvent B to 0% solvent A/100% solvent B; 28.6–31 min, isocratic elution, 0% solvent A/100% solvent B; 31–34 min, isocratic elution, 60% solvent A/40% solvent B. Eight electrode detector potentials (0–700 mV in increments of 100 mV) were used to measure the amount of flavanones.

Distribution of flavanones after transport experiments. Flavanones were loaded on the apical side of the cell monolayer for the desired incubation time at 37 °C. The levels of flavanone in the apical and basolateral solutions were then measured. The monolayer cells were rinsed with HBSS (pH 6.0 or 7.4) and extracted with methanol/solvent A (10:1, v/v) containing 1% Triton X-100 for 60 min. Flavanones in this extract were measured and used as an estimate of flavanone uptake by the Caco-2 cells.

Data analysis. The slope of the initial linear portion of the curve of the amount transported (nmol/min/mg protein) *versus* time (min), calculated by linear regression analysis, was defined as the permeation rate (nmol/min/mg protein), J. All the results are expressed as the means ± SD. A statistical analysis was conducted by Dunnett's test between the control and test groups.

Results and discussion

A representative chromatogram of flavanones transported into the basolateral solution is presented in Fig. 1. All flavanones were determined at a detection limit of <0.5 pmol on the column. Purity of each peak was assessed using the accuracy of the ratio of the peak area for the adjacent oxidation channels (lower or upper) to that of the dominant oxidation channel. Greater than 70% ratio accuracy was regarded as peak purity [17]. The dominant oxidation potentials and retention times were indicated as followed: hesperetin (400 mV, 17.5 min), naringenin (600 mV, 15.8 min), and eriodictyol (200 mV, 11.9 min).

We initially examined whether the H^+-coupled active transport responsible for the absorption of hesperetin [1] might also be involved in the transport of other flavanones. Thus the cell permeability of flavanones, such as naringenin and eriodictyol, was measured using Caco-2 cell monolayers, and simultaneously compared with that of hesperetin. In our previous study, which compared the absorption of hesperetin and hesperidin, the concentration of these compounds was set at 0.5 mM [1]. However, because of the low solubility of hesperetin, naringenin and eriodictyol in HBSS, their absorption characteristics were examined at 50 μM in Caco-2 cells. Permeation rates (nmol/min/mg protein) were shown in Fig. 2. The absorption profiles of hesperetin, naringenin, and eriodictyol at 50 μM were similar to that of hesperetin at 0.5 mM in the presence and absence of a proton gradient (Fig. 2 and Tables 1, 2).

After loading naringenin or eriodictyol on the apical side of monolayer in the absence of a proton gradient,

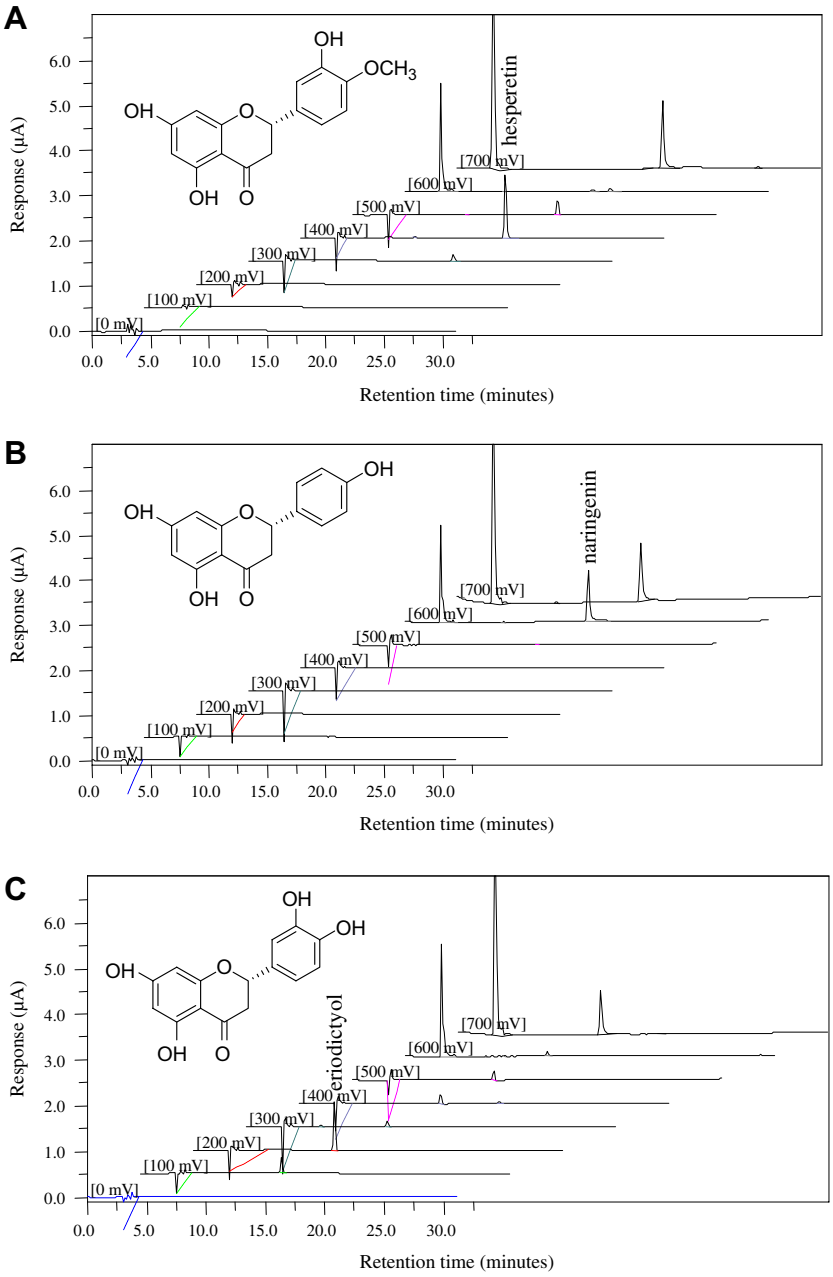

Fig. 1. Chemical structures and the results of HPLC chromatography coupled with coulometric array detection of hesperetin (A), naringenin (B) and eriodictyol (C) transported across Caco-2 cell monolayers.

60% and 78%, respectively, remained on the apical side, with 38% and 21%, respectively, being transported to the basolateral side. In the presence of a proton gradient, 62% naringenin and 47% eriodictyol of apically loaded compound was transported to the basolateral side, and only 31% naringenin and 47% eriodictyol remained on the apical side (Table 2). As illustrated in Fig. 2D and E, $J_{ap \rightarrow bl}$ of both naringenin and eriodictyol remained almost constant irrespective of the TER. These findings indicate that naringenin and eriodictyol permeates Caco-2 cell monolayers *via* transcellular routes.

Fig. 2F and G show the relationship between the initial permeation rate of naringenin (F) or eriodictyol (G) and their concentration in the presence of a proton gradient. Flavanones are slightly solved in water, however they are soluble up to 0.5 mM in 1% DMSO. Naringenin and eriodictyol showed concentration-dependent and non-saturable transport at the range of 0.02–0.5 mM.

The $J_{ap \rightarrow bl}$ of hesperetin, naringenin and eriodictyol were 4- to 7-fold higher than that of $J_{bl \rightarrow ap}$ in the presence of a proton gradient (Fig. 2A–C), respectively. Apically loaded NaN_3 inhibited the $J_{ap \rightarrow bl}$ by approximately 50% (Table 1), suggesting that H^+-coupled active transport and transcellular passive diffusion contributes to the absorption of flavanones across the intestinal epithelium by almost the same extent. Indeed, the $J_{ap \rightarrow bl}$ and $J_{bl \rightarrow ap}$

of hesperetin, naringenin, and eriodictyol in the absence of proton gradient were also about half the $J_{ap \rightarrow bl}$ of each flavanone in the presence of a proton gradient (Fig. 2A–C). The $J_{ap \rightarrow bl}$ in the presence of proton gradient, increases in the following order; naringenin > hesperetin > eriodictyol. These observations might be attributed to the greater efficiency of absorption or bioavailability of naringenin over hesperetin, which has been reported in several studies [5,6].

Specifically, our results show that absorption of all three substrates is characterized by: (i) H^+-driven polarized transport in the apical-to-basolateral direction (ii) paracel-

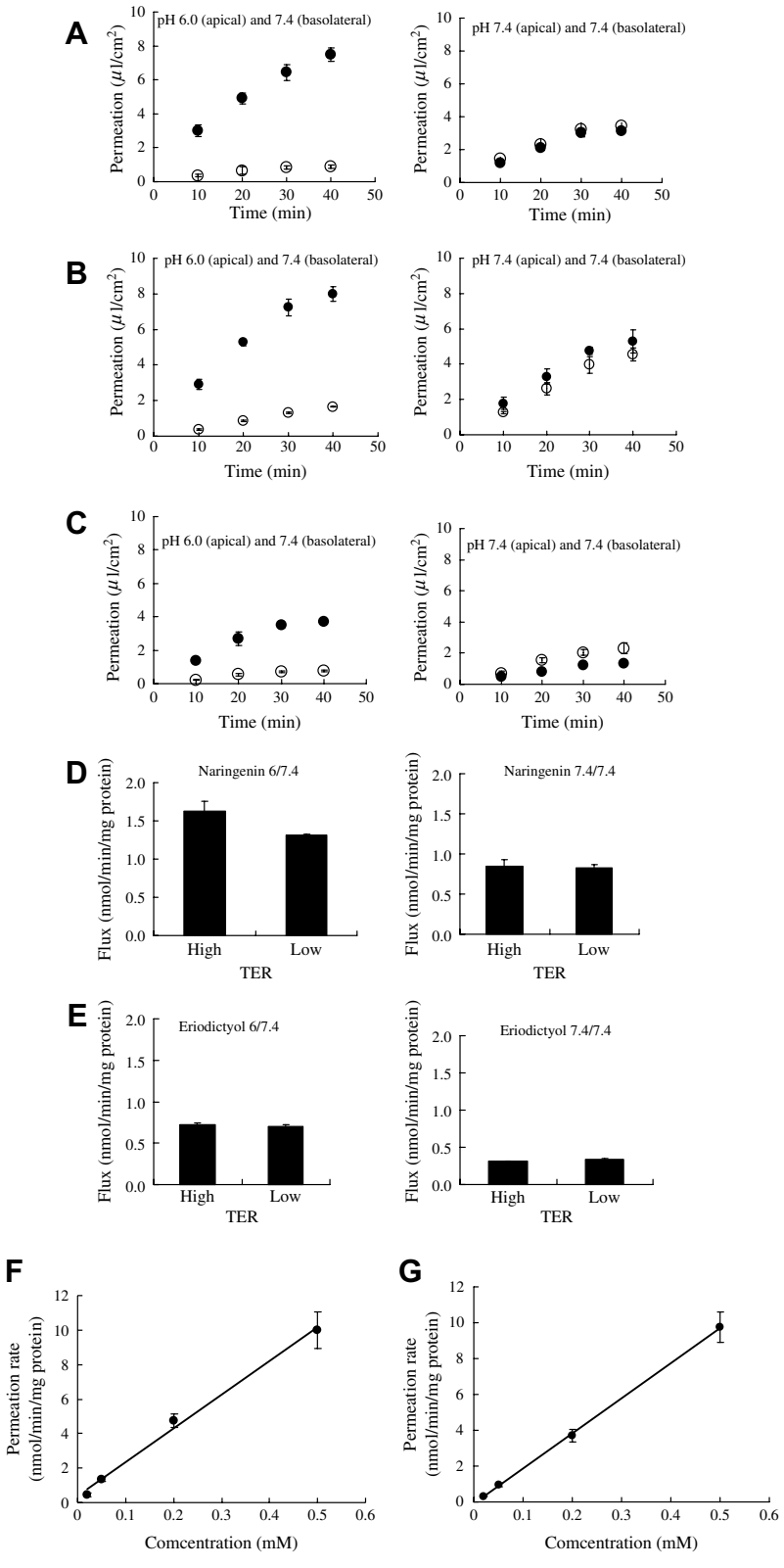

Table 1
Effects of NaN_3, benzoic acid, ferulic acid, DIDS, and ibuprofen on the permeation of hesperetin, naringenin, and eriodictyol in Caco-2 cells in the presence of a proton gradient

	Relative permeation (% of control)		
	Hesperetin	Naringenin	Eriodictyol
NaN_3	$56.81 \pm 3.27^*$	$50.16 \pm 5.60^*$	$55.26 \pm 2.51^*$
Benzoic acid	$72.64 \pm 9.45^*$	$69.69 \pm 4.92^*$	$65.98 \pm 1.98^*$
Ferulic acid	108.9 ± 12.70	125.4 ± 12.50	103.6 ± 6.20
DIDS[a]	109.7 ± 2.31	101.6 ± 4.81	96.8 ± 16.94
Ibuprofen	100.6 ± 1.5899	99.5 ± 3.66	103.5 ± 0.77

The amount of hesperetin, naringenin, and eriodictyol transported in apical-to basolateral direction was measured after incubation at 37 °C for 40 min in the absence or presence of NaN_3, benzoic acid, and ferulic acid at 10 mM, DIDS at 2 mM, and ibuprofen at 5 mM, respectively. (Apical pH, 6.0; basolateral pH, 7.4.) All compounds were apically loaded.
Data are expressed as a percentage of the control and are presented as means ± SD of three or more experiments. *Significantly lower than the control value ($P < 0.01$).
[a] 4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid.

Table 2
Distribution of naringenin and eriodictyol after transepithelial transport experiments in the presence or absence of a proton gradient

	Ap	Cell	Bl
Distribution (%)			
Naringenin (6.0/7.4)	31.26 ± 0.79	6.84 ± 0.63	61.90 ± 1.38
Naringenin (7.4/7.4)	59.64 ± 1.73	2.19 ± 0.18	38.17 ± 1.63
Eriodictyol (6.0/7.4)	47.26 ± 3.17	5.96 ± 2.10	46.77 ± 2.23
Eriodictyol (7.4/7.4)	78.37 ± 2.65	0.85 ± 0.20	20.78 ± 2.45

After naringenin or eriodictyol was loaded apically, transepithelial transport experiments were done and distributions were examined both in the presence and absence of a proton gradient (apical side, pH 6.0 or 7.4; basolateral side, pH 7.4).
Each value represents means ± SD of three experiments.

lular permeability-independent non-saturable transport (iii) preferable distribution into the basolateral side (iv) enhanced intracellular accumulation of apically loaded compound in the presence of H^+ gradient (v) significant inhibition of each $J_{ap \rightarrow bl}$ by benzoic acid, a substrate of MCT. However, the precise mechanism remains obscure. Firstly, our results clearly shows that the H^+-coupled active transport, recently reported to be responsible for absorption of hesperetin [1], is commonly involved in the absorption of flavanones (Fig. 3).

Secondly, to investigate the proton-coupled transport characteristics of flavanones further, we examined the effects of various inhibitors or substrates on the $J_{ap \rightarrow bl}$ of each flavanones (Table 1). A metabolic inhibitor, NaN_3 (10 mM) markedly reduced the transport of hesperetin, naringenin, and eriodictyol. Benzoic acid (10 mM), which is a substrate of the MCT, caused a slight reduction in the transport of flavanones. By contrast, ferulic acid (10 mM), a substrate for a discrete class of MCTs from those that recognize benzoic acid [15], and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), an anion-exchanger inhibitor (2 mM) did not affect $J_{ap \rightarrow bl}$ of flavanones. Furthermore, ibuprofen (5 mM), a substrate of the Na^+/monocarboxylate transporter (SMCT) [18], did not affect $J_{ap \rightarrow bl}$ of hesperetin, naringenin and eriodictyol (Table 1). Until now, according to a series of *in vitro* and *in vivo* studies concerning with absorption and bioavailability of PAs, it was apparent that a H^+-driven MCT-mediated transport operates during the absorption and distribution of PAs such as ferulic, *p*-coumaric, or caffeic acids, 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C) [9,15,16,19–21]. However, the extent of the contribution of each mechanism to the total absorption across the epithelium differs according to the affinity for MCT and the lipophilicity of the corresponding compound. Furthermore, microbial metabolites of poorly absorbed polyphenols, the possible candidates of bioactive compounds *in vivo* of parent polyphenols, are also absorbed and distributed by this H^+-driven MCT in the same manner as the case of PAs [22,23]. Thus, these recent findings highlight the profound significance of this H^+-coupled transport system on health benefits, and lead to the concept of novel dietary nutrients, known as "metabo-nutrients", which are absorbed and distributed throughout the body and exert certain health effects in humans *via* this transport system [24]. From the results that only benzoic acid have inhibitory effects on $J_{ap \rightarrow bl}$ of flavanones in Table 1, the H^+-coupled active transport for flavanones, appears to be different from that for PAs, and SMCT-or anion exchanger-mediated transport. This is well consistent with the result that ferulic acid also have no stimulatory effect on $J_{ap \rightarrow bl}$ of flavanones in Table 3. Nevertheless, *trans*-stimulation with benzoic acid also exhibited no effects of flavanone transport (Table 3), suggesting that benzoic acid would be an effective inhibitor not but a substrate of this

Fig. 2. Characteristics of the transepithelial permeation of hesperetin, naringenin, and eriodictyol across Caco-2 cell monolayers. Transport of hesperetin (A), naringenin (B), and eriodictyol (C) (50 μM) from the apical side to the basolateral side (●) and from the basolateral side to the apical side (○) was measured at 37 °C in both the presence (left panel) and absence (right panel) of a proton gradient (apical pH, 6.0 or 7.4; basolateral pH, 7.4). Permeation rates (nmol/min/mg protein) were indicated as followed: (A) hesperetin 6.0/7.4 ($J_{ap \rightarrow bl}$: 0.76 ±0.11, $J_{bl \rightarrow ap}$: 0.12 ± 0.01), hesperetin 7.4/7.4 ($J_{ap \rightarrow bl}$: 0.34 ± 0.06, $J_{bl \rightarrow ap}$: 0.34 ± 0.07), (B) naringenin 6.0/7.4 ($J_{ap \rightarrow bl}$: 1.07 ± 0.05, $J_{bl \rightarrow ap}$: 0.23 ± 0.01), naringenin 7.4/7.4 ($J_{ap \rightarrow bl}$: 0.74 ± 0.04, $J_{bl \rightarrow ap}$: 0.67 ± 0.10), (C) eriodictyol 6.0/7.4 ($J_{ap \rightarrow bl}$: 0.66 ± 0.08, $J_{bl \rightarrow ap}$: 0.09 ± 0.05), eriodictyol 7.4/7.4 ($J_{ap \rightarrow bl}$: 0.26 ± 0.04, $J_{bl \rightarrow ap}$: 0.32 ±0.01). Naringenin (D) or eriodictyol (E) (50 μM) was loaded on the apical side, and the flux from the apical side to the basolateral side was measured at 37 °C in the presence and absence of a proton gradient (apical pH, 6.0 or 7.4; basolateral pH, 7.4). TER values are indicated as follows: (D) naringenin 6/7.4 high: 750.0 ± 47.0, low: 136.3 ± 5.86. Naringenin 7.4/7.4 high: 1002.7 ± 45.0, low: 140.0 ± 6.1. (E) Eriodictyol 6/7.4 high: 626.0 ± 26.0, low: 181.0 ± 4.6. Eriodictyol 7.4/7.4 high: 695.0 ± 35.0, low: 180.7 ± 6.3. Concentration dependence of naringenin (F) and eriodictyol (G) transport across Caco-2 cells in the presence of a proton gradient (apical pH, 6.0; basolateral pH, 7.4). Each point is the mean ± SD of three experiments.

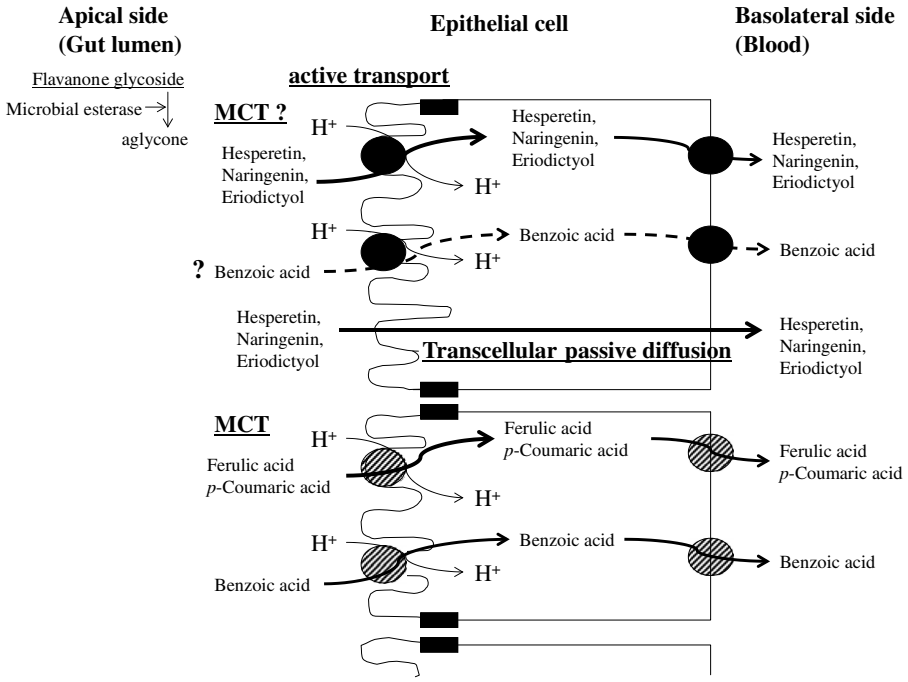

Fig. 3. Possible routes for absorption of flavanones and phenolic acids.

Table 3
Trans-stimulation of flavanone permeability by preloaded ferulic acid and benzoic acid in Caco-2 cells in the presence of a proton gradient

	Relative permeation (% of control)	
	Ferulic acid	Benzoic acid
Hesperetin	100.5 ± 7.60	97.56 ± 2.95
Naringenin	91.55 ± 8.95	91.24 ± 1.38
Eriodictyol	110.8 ± 4.84	100.7 ± 4.15

The Caco-2 cells were preloaded by incubation with 10 mM ferulic acid and benzoic acid for 20 min at 37 °C. Apical-to-basolateral permeability was measured in the presence of a proton gradient (apical pH, 6.0; basolateral pH, 7.4).
Data are expressed as a percentage of the control and are presented as means ± SD of three or more experiments.

H^+-coupled active transport. Indeed, Shim et al. [25] have reported that despite the naringenin might not be a substrate of MCT1, it exhibited strong inhibitory effect on MCT1. Several proton-coupled transporters, like MCT and PEPT1, have been identified and partially characterized in small intestine [26,27]. However, PEPT1 does not appear to be involved in the absorption of flavanones because Gly-Sar failed to inhibit their transport [1]. It is apparent that various subtypes of MCT, such as MCT1, MCT3, MCT4, MCT5, and MCT6, are expressed in Caco-2 cells [28]. As for the transport for PAs, the possible involvement of a novel MCT subtype other than MCT1–MCT4 has also been reported [29]. In order to fully assess the impact of polyphenols on human health, further studies are required to establish those transport system molecularly in detail.

In conclusion, it have been verified that flavanones such as naringenin, eriodictyol, and hesperetin, are commonly absorbed by both transcellular passive diffusion and proton-driven active transport other than MCT-mediated transport for PAs, SMCT- or anion exchanger-mediated transport. To fully understand and assess the health effects of dietary flavonoids and PAs, it is desirable to characterize the relevant active transport systems at the molecular level and determine their tissue distribution and subcellular localization. These studies are currently underway in our laboratory.

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