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Transepithelial transport of hesperetin and hesperidin in intestinal Caco-2 cell monolayers

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Abstract

The cell permeability of hesperetin and hesperidin, anti-allergic compounds from citrus fruits, was measured using Caco-2 monolayers. In the presence of a proton gradient, hesperetin permeated cells in the apical-to-basolateral direction at the rate ($J_{ap\rightarrow bl}$) of 10.43±0.78 nmol/min/mg protein, which was more than 400-fold higher than that of hesperidin (0.023±0.008 nmol/min/mg protein). The transepithelial flux of hesperidin, both in the presence or absence of a proton gradient, was nearly the same and was inversely correlated with the transepithelial electrical resistance (TER), indicating that the transport of hesperidin was mainly via paracellular diffusion. In contrast, the transepithelial flux of hesperetin was almost constant irrespective of the TER. Apically loaded NaN_3 or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) decreased the $J_{ap\rightarrow bl}$ of hesperetin, in the presence of proton gradient, by one-half. In the absence of a proton gradient, both $J_{ap\rightarrow bl}$ and $J_{bl\rightarrow ap}$ of hesperetin were almost the same (5.75±0.40 and 5.16±0.73 nmol/min/mg protein). $J_{bl\rightarrow ap}$ of hesperetin in the presence of a proton gradient was lower than $J_{bl\rightarrow ap}$ in the absence of a proton gradient. Furthermore, $J_{bl\rightarrow ap}$ in the presence of a proton gradient remarkably increased upon addition of NaN_3 specifically to the apical side. These results indicate that hesperetin is absorbed by transcellular transport, which occurs mainly via proton-coupled active transport, and passive diffusion. Thus, hesperetin is efficiently absorbed from the intestine, whereas hesperidin is poorly transported via the paracellular pathway and its transport is highly dependent on conversion to hesperetin via the hydrolytic action of microflora. We have given novel insight to the absorption characteristics of hesperetin, that is proton-coupled and energy-dependent polarized transport.

Keywords: Hesperetin; Hesperidin; Transcellular active transport; Transcellular passive diffusion; Caco-2

1. Introduction

Citrus fruits, such as orange (*Citrus sinensis*) and grapefruit (*Citrus paradisi*), contain various typical flavonoids such as flavanone, flavanone glycoside, and polymethoxyflavone [1,2]. These have recently received attention because of their potential therapeutic benefits including anti-allergic [3,4], anti-oxidant [5], and anti-cancer [6,7] effects, as well as prevention of bone loss [8] and anti-inflammatory effects [9,10]. Of the flavonoids contained in citrus fruit, flavanones are the most abundant. The predominant flavanone in grapefruit is naringin and in oranges, hesperidin. These flavanones occur only rarely in other plants [11]. In a recent study on the polyphenol intake in Finland, the most highly consumed flavonoid was hesperidin (28.3 mg/day), representing 30% of the total flavonoid intake [12]. Hesperidin (formerly called 'vitamin P') was found to be essential for maintaining the permeability and integrity of the microvascular endothelium [13]. Our previous study on anti-allergic constituents of *Citrus unshiu* found that hesperetin (3′,5,7-trihydroxy-

Abbreviations: AC, altepillin C; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ECD, electrochemical detector; Gly-Sar, glycylsarcosine; HBSS, Hanks' balanced salt solution; PEPT, peptide transporter; SGLT, sodium-dependent D-glucose cotransporter; TER, transepithelial electrical resistance; MCT, monocarboxylic acid transporter; MRP, multidrug resistance-associated protein

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4′-methoxyflavanone) (Fig. 1A) and nobiletin (3′,4′,5,6,7,8-hexamethoxyflavone) but not hesperidin (hesperetin glycoside) have potent inhibitory activities against chemical mediator release from rat basophilic leukemia RBL2H3 cells [3]. Additionally, citrus fruits contain only a very low amount of hesperetin (hesperidin aglycone), and most of the flavanones exist as glycosides. The finding of Park et al. [4] that orally but not intraperitoneally administered hesperidin inhibited passive cutaneous anaphylaxis (PCA) in mice can be explained by the fact that the former is hydrolyzed by microflora β-glucosidases to the immunologically active hesperetin (hesperidin aglycone) before intestinal absorption [14,15]. Although the anti-allergic benefit of hesperetin and its glucoside has been suggested, very little is known about the bioavailability of these natural products.

Metabolism and intestinal absorption determines the bioavailability of flavonoids. Generally, flavonoids are absorbed after transport by passive diffusion and attachment to the lipid bilayer of the intestinal epithelial cell surface [16,17]. It has been reported that, in human, isoflavone aglycones are transported into enterocytes more efficiently than their glucoside counterparts because of their moderate lipophilicity [16] and easier absorption [18]. The same properties appear to govern the transport of quercetin and its glucosides [17]. Quercetin 4′-β-glucoside might be taken up intracellularly by SGLT1 (sodium-dependent D-glucose cotransporter-1) [19] and pumped back out into the intestinal lumen by MRP2 (multidrug resistance-associated protein-2) [20] in Caco-2 cells. Thus the absorption and metabolism of flavonoids are complex. Nevertheless, it is reasonable to assume that flavonoids cross epithelial cell membranes by transcellular passive diffusion [16,21]. Indeed, a precise analysis of the mechanisms of gastrointestinal absorption of flavonoids and their glycosides, such as hesperetin and hesperidin, has not been performed.

In this study, we investigate and clarify the absorption mechanism of hesperetin and hesperidin using Caco-2 cells as an *in vitro* model of intestinal absorption and metabolism [22,23].

2. Materials and methods

2.1. 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), nonessential amino acids, penicillin, streptomycin, gentamycin, and Hanks' balanced salt solution (HBSS) were all from Invitrogen

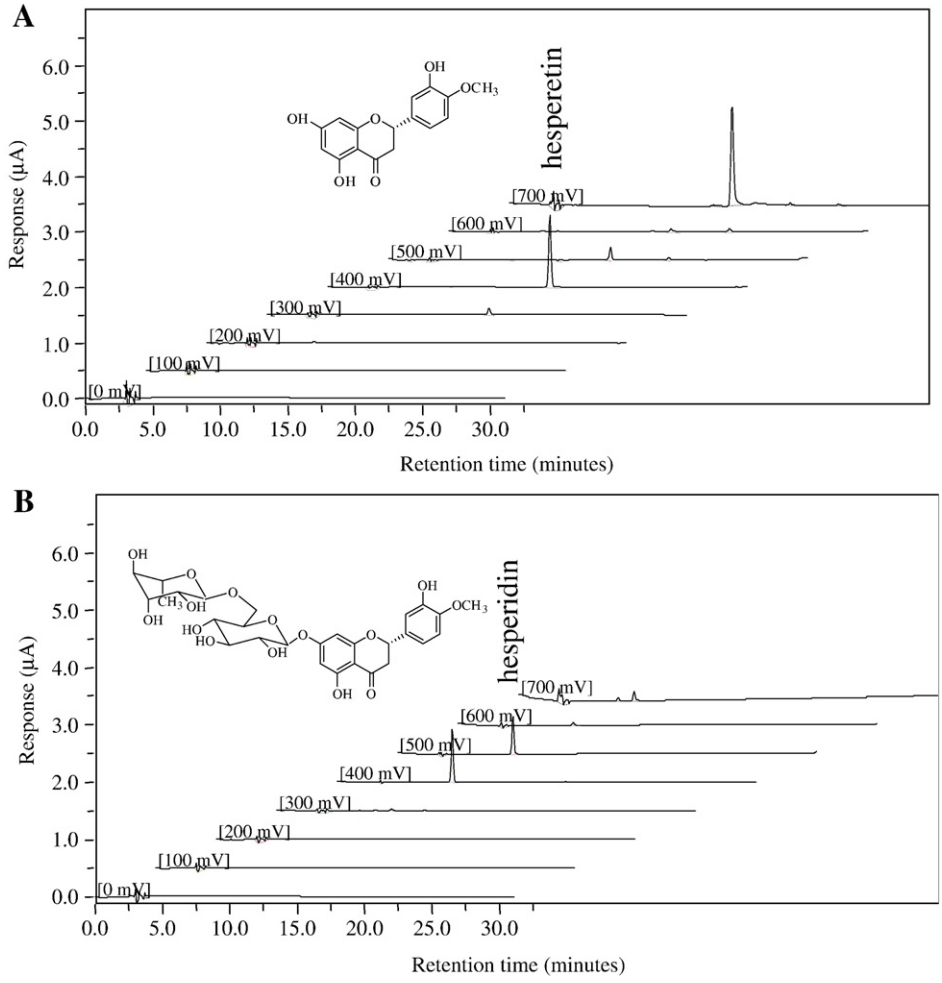


Fig. 1. Chemical structures and the results of high performance liquid chromatography coupled with coulometric array detection of hesperetin (A) and hesperidin (B) transport across Caco-2 cell monolayers.

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 and hesperidin were from Wako Pure Chemicals Inc., Ltd. (Osaka, Japan). All other chemicals used in this study were of analytical grade.

2.2. Cell culture

Caco-2 cells were cultured in DMEM containing 20% FCS, 1% nonessential 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 50 to 90 were used.

2.3. Transepithelial transport experiments

Cells were normally grown in 75-cm^2 tissue culture dishes to confluence and 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 cells were rinsed gently three times with HBSS and left for equilibration 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 [24]. Before and after the transport experiment, the TER of the monolayer was also measured.

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 hesperetin or hesperidin (0.5 mmol/l) was added to the apical side. Flavanones were dissolved in dimethyl sulfoxide (DMSO) and diluted with HBSS before experiments. The resulting final concentration of DMSO, 1%, did not affect the transport [25]. After the desired incubation time at 37 °C, the basolateral solution was collected and then replaced with an equal volume of HBSS. Na^+-free ($-Na^+$) HBSS was prepared by replacing NaCl with choline Cl and omitting NaH_2PO_4. The amount of hesperetin or hesperidin 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 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.

2.4. Chromatographic conditions

HPLC-ECD fitted with a coulometric detection system was used for analysis as previously reported [26,27]. 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 to 700 mV in increments of 100 mV) were used to measure the amount of hesperetin and hesperidin.

2.5. Distribution of hesperetin and hesperidin after transport experiments

Hesperetin and hesperidin were loaded on the apical or basolateral 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.

2.6. 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 ± S.D. A statistical analysis was conducted by Dunnett's test between the control and test groups.

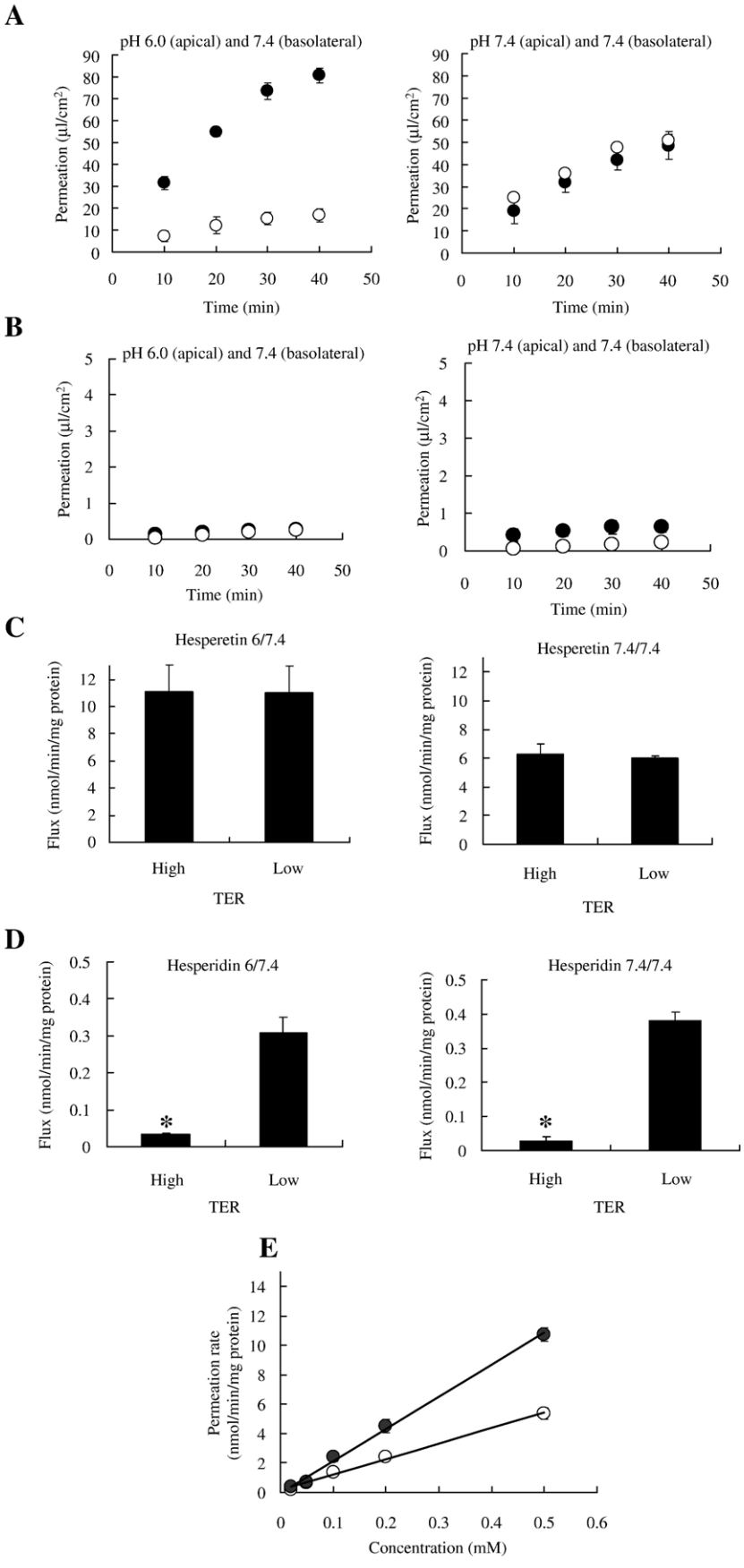
3. Results

3.1. HPLC analysis of hesperetin and hesperidin transported across Caco-2 cell monolayers

A representative chromatogram of hesperetin and hesperidin transported into the basolateral solution is presented in Fig. 1. Both flavanones were determined at a detection limit of <0.5 pmol on the column. The results were reproducible without requiring any sample pre-treatment. 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. The voltammetric response of the analyte across these channels was unique for each compound. Greater than 70% ratio accuracy was regarded as peak purity [28]. The dominant oxidation potential was 400 mV and the retention times (RT) for hesperetin and hesperidin were 16.5 min and 8.57 min, respectively.

3.2. Characteristics of transepithelial transport of hesperetin and hesperidin

Bi-directional transport of hesperetin and hesperidin (0.5 mM) across a Caco-2 cell monolayer was examined in the presence or absence of an inwardly directed proton gradient (Fig. 2A, B). In the presence of a proton gradient (an apical pH, 6.0 and basolateral pH, 7.4), the permeation rate of hesperetin in the apical to the basolateral direction, $J_{ap \rightarrow bl}$ (10.43 ± 0.78 nmol/min/mg protein) was greater than the $J_{bl \rightarrow ap}$ (2.02 ± 0.24 nmol/min/mg protein) in the opposite direction. In the absence of a proton gradient (apical pH, 7.4; basolateral pH, 7.4), $J_{ap \rightarrow bl}$ (5.75 ± 0.40 nmol/min/mg protein) was almost the same as $J_{bl \rightarrow ap}$ (5.16 ± 0.73 nmol/min/mg protein), but only half the value of $J_{ap \rightarrow bl}$ in the presence of a proton gradient. Hesperetin exhibits polarized transport in the presence of a proton gradient. Furthermore, $J_{bl \rightarrow ap}$ in the presence of proton gradients was lower than any other permeation rate (2.02 ± 0.24 nmol/min/mg protein). These results indicate that the transport of hesperetin is complex. By contrast, the permeation rates of hesperidin, $J_{ap \rightarrow bl}$ and $J_{bl \rightarrow ap}$, in the presence and absence of proton gradient (0.023–0.044 nmol/min/mg protein) were nearly the same, suggesting that hesperidin moved through the paracellular space (Fig. 2B). The permeation rate of hesperidin $J_{ap \rightarrow bl}$ in the presence of a proton gradient was more than 400-fold lower than that of hesperetin.


A
pH 6.0 (apical) and 7.4 (basolateral)
pH 7.4 (apical) and 7.4 (basolateral)
Permeation (μl/cm²)
Time (min)
B
pH 6.0 (apical) and 7.4 (basolateral)
pH 7.4 (apical) and 7.4 (basolateral)
Permeation (μl/cm²)
Time (min)
C
Hesperetin 6/7.4
Hesperetin 7.4/7.4
Flux (nmol/min/mg protein)
High
Low
TER
D
Hesperidin 6/7.4
Hesperidin 7.4/7.4
Flux (nmol/min/mg protein)
*
High
Low
TER
E
Permeation rate (nmol/min/mg protein)
Concentration (mM)

TER values after the hesperetin transport experiment were as followed: in the presence of the proton gradient ($J_{ap\rightarrow bl}$, 637±37.7; $J_{bl\rightarrow ap}$, 587±30.4); in the absence of the proton gradient ($J_{ap\rightarrow bl}$, 531±52.7; $J_{bl\rightarrow ap}$, 611±51.6). TER values after the hesperidin transport experiment were as followed: in the presence of the proton gradient ($J_{ap\rightarrow bl}$, 536±12.7; $J_{bl\rightarrow ap}$, 562±28.0); in the absence of the proton gradient ($J_{ap\rightarrow bl}$, 631±32.0; $J_{bl\rightarrow ap}$, 654±11.6). Although the addition of CCCP (proton-ionophore) gave a slight decrease of TER values, TER values at the end of each transport experiment were kept >300 Ω. It was, thus, judged that the TER did not change during the incubation, and that hesperetin permeation was not affected by the incubation. To obtain Caco-2 cell monolayers with different TER values for transport study, cytochalasin D (0.1 mM) was added to the apical side. The apical-to-basolateral transport of hesperetin and hesperidin through these monolayers was then examined in the presence or absence of a proton gradient. As illustrated in Fig. 2C and D, the transepithelial flux of hesperidin was inversely correlated with the TER, whereas that of hesperetin remained almost constant irrespective of the TER. This finding indicates that hesperidin and hesperetin permeates Caco-2 cell monolayers via paracellular and transcellular routes, respectively.

To investigate the transport characteristics responsible for proton-coupled polarized transport of hesperetin, we added hesperetin to the apical chamber in the presence of a proton gradient and examined the effects of various inhibitors or substrates on permeation (Table 1A). Both a metabolic inhibitor, NaN_3 (10 mM), and proton-ionophore (CCCP, 2 mM), markedly reduced the transport of hesperetin. However, inhibitor for the monocarboxylic acid transporter (MCT), phloretin (0.3 mM), and the substrate of the H^+-coupled peptide transporter 1 (PEPT1), glycylsarcosine (Gly-Sar, 10 mM), did not affect hesperetin transport. Higher concentration of phloretin could not be examined because of noticeable cytotoxicity [29]. Benzoic acid (10 mM), which is the substrate of the MCT, slightly reduced the hesperetin transport. Furthermore, the Na^+ dependence of hesperetin transport was examined. The relative $J_{ap\rightarrow bl}$ (percent of control) of hesperetin under Na^+-free conditions was almost the same both in the presence (103.3±6.91% of control) and absence (102.3±14.00% of control) of the proton gradient, indicating that the hesperetin transport was independent of Na^+.

The effect on transport of adding NaN_3 to either the apical or basolateral solution in the presence of a proton gradient was studied. The $J_{bl\rightarrow ap}$ of hesperetin increased about two-fold level as control when NaN_3 was loaded apically (Table 1B). In contrast, the $J_{bl\rightarrow ap}$ of hesperetin was unaffected by the basolateral addition of NaN_3.

Table 1
Effects of various compounds on hesperetin transport (A) and effects of NaN_3 and CCCP loaded apically or basolaterally on the permeation of hesperetin loaded basolaterally (B) in Caco-2 cells in the presence of a proton gradient

	Relative permeation (percent of control)
(A) $J_{ap\rightarrow bl}$ of hesperetin	
NaN_3 (10 mM)	40.4±6.70*
CCCP[a] (2 mM)	59.2±3.60*
Gly-Sar[b] (10 mM)	94.1±8.19
Benzoic acid (10 mM)	65.8±3.04*
Phloretin (0.3 mM)	97.7±4.11
(B) $J_{bl\rightarrow ap}$ of hesperetin	
NaN_3ap (10 mM)	190.2±11.2*
NaN_3bl (10 mM)	115.3±3.75
CCCPap (2 mM)	149.6±9.16*

(A) The amount of hesperetin transported in apical-to-basolateral direction was measured after incubation at 37 °C for 40 min in the absence or presence of each compound (apical pH, 6.0; basolateral pH, 7.4). All compounds were apically loaded.
(B) Transepithelial transport experiments were done as described in Materials and methods. Basolateral-to-apical permeability was measured in the presence of a proton gradient (apical pH, 6.0; basolateral pH, 7.4) with or without (control) NaN_3 and CCCP, loaded apically or basolaterally.
NaN_3ap, NaN_3 apically loaded; NaN_3bl, NaN_3 basolaterally loaded; CCCPap, CCCP apically loaded.
Data are expressed as a percentage of the control and are presented as the mean± S.D. of three or more experiments. *Significantly lower than the control value ($P<0.01$).
[a] Carbonyl cyanide *m*-chlorophenylhydrazone.
[b] Glycylsarcosine.

3.3. Concentration dependence of hesperetin transport

Fig. 2E shows the relationship between the initial permeation rate of hesperetin and its concentration in the presence or absence of a proton gradient. Because hesperetin is hardly soluble in water, it was dissolved with 1% DMSO. This concentration of DMSO did not produce any significant alteration in transcellular and paracellular permeability [25]. It was shown that hesperetin permeated non-saturably and linearly in concentration-dependent manner at the range of 0.02–0.5 mM (Fig. 2E). At all the concentrations tested, the permeation rate in the absence of a proton gradient was half of that in the presence of a proton gradient.

Fig. 2. Characteristics of the transepithelial transport of hesperetin and hesperidin across Caco-2 cell monolayers. (A and B) Transport of hesperetin (A) and hesperidin (B) (0.5 mM) 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 follows: (A) hesperetin 6.0/7.4 ($J_{ap\rightarrow bl}$: 10.43±0.78, $J_{bl\rightarrow ap}$: 2.02±0.24), hesperetin 7.4/7.4 ($J_{ap\rightarrow bl}$: 5.75±0.40, $J_{bl\rightarrow ap}$: 5.16±0.73), (B) hesperidin 6.0/7.4 ($J_{ap\rightarrow bl}$: 0.023±0.008, $J_{bl\rightarrow ap}$: 0.039±0.007), hesperidin 7.4/7.4 ($J_{ap\rightarrow bl}$: 0.044±0.022, $J_{bl\rightarrow ap}$: 0.031±0.004). (C and D) Hesperetin (C) or hesperidin (D) (0.5 mM) 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: (C) hesperetin 6/7.4 high: 594±1.0, low: 244±8.3. hesperetin 7.4/7.4 high: 597±9.9, low: 221±8.1. (D) hesperidin 6/7.4 high: 605±12, low: 259±6.4. hesperidin 7.4/7.4 high: 575±16, low: 243±9.1. (E) Concentration dependence of hesperetin transport across Caco-2 cells in the presence (●) and absence (○) of a proton gradient. Each point is the mean±S.D. of three experiments. *Significantly lower than the control value ($P<0.01$).

Table 2
Distribution of hesperetin and hesperidin after transepithelial transport experiments in the presence or absence of a proton gradient (A) and effects of NaN_3 and CCCP loaded apically or basolaterally on the distribution of hesperetin loaded basolaterally (B) in Caco-2 cell monolayers in the presence of a proton gradient

	Ap	Cell	Bl
(A) Distribution (%)			
Hesperetin(6.0/7.4)	25.5±3.28	4.14±0.88	70.4±3.92
Hesperetin(7.4/7.4)	75.2±2.86	1.62±0.27	23.1±2.84
Hesperidin(6.0/7.4)	99.3±0.17	0.34±0.16	0.32±0.03
Hesperidin(7.4/7.4)	98.6±0.40	0.85±0.37	0.58±0.08
(B) Distribution (%)			
Control	4.13±0.65	2.26±0.37	93.6±0.82
NaN_{3ap}	7.09±2.19	2.40±0.44	90.5±1.77
NaN_{3bl}	4.39±0.85	1.73±0.21	93.9±0.79
$CCCP_{ap}$	6.84±0.15	2.19±0.32	91.0±0.44

(A) After hesperetin or hesperidin 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).
(B) After hesperetin was loaded basolaterally, transepithelial transport and distribution were examined in the presence of a proton gradient (apical pH, 6.0; basolateral pH, 7.4) with or without (control) NaN_3 (10 mM) and CCCP (2 mM), loaded apically or basolaterally.
NaN_3ap, NaN_3 apically loaded; NaN_{3bl}, NaN_3 basolaterally loaded; CCCPap, CCCP apically loaded.
Each value represents the mean±S.D. of three experiments.

3.4. Distribution of hesperetin in Caco-2 cell monolayers

After hesperetin was loaded on the apical side of monolayers in the absence of a proton gradient, 75% of apically loaded hesperetin was remained in the apical side, and 23% had been transported to the basolateral side. In the presence of a proton gradient, 70% of apically loaded hesperetin had been transported to the basolateral side, and only 23% was remained in the apical side (Table 2A). On the other hand, regardless of the presence or absence of proton gradient, >98% of hesperidin added to the apical side remained in apical side (Table 2A).

After hesperetin was loaded on the basolateral side of monolayers in the presence of a proton gradient, levels of hesperetin were high both intracellularly (2.26%) and basolaterally (93.6%) but low apically (4.13%) (Table 2B). The apical addition of NaN_3 or CCCP to hesperetin in the presence of a proton gradient caused hesperetin level to increase in apical fraction and decrease in the basolateral fraction (Table 2B). Basolaterally loaded NaN_3 did not affect the distribution of hesperetin in the presence of a proton gradient (Table 2B).

4. Discussion

In our previous study, we have demonstrated that hydroxycinnamic acid derivatives and colonic microbial metabolites of flavonoids, such as ferulic acid, *p*-coumaric acid, caffeic acid, artepillin C (AC), and hydroxylated phenyl propionic or acetic acids were transported in Caco-2 cells via an MCT to the some extent [26,27,30–33]. Of these compounds, ferulic acid (1 mM) in the presence of a proton gradient had the highest $J_{ap\rightarrow bl}$ (9.79±0.43 nmol/min/mg protein). Because the solubility of hesperetin and hesperidin is low in HBSS, a lower concentration of these compounds (0.5 mM) was used to examine their transport across Caco-2 monolayers. The $J_{ap\rightarrow bl}$ of hesperetin (10.43±0.78 nmol/min/mg protein) was higher than that of FA, even though the concentration of hesperetin was lower than that of FA, indicating that efficiency of hesperetin absorption in Caco-2 cells is higher than that of FA (Fig. 2A). Quercetin and its glucosides are glucuronidated during transport through the Caco-2 monolayers [17,34]. When the transported samples on the basolateral side were treated by the deconjugating enzyme sulfatase H-5, containing both sulfatase and glucuronidase, no change in the hesperetin content was apparent (data not shown). Thus conjugation of hesperetin during permeation was unlikely, at least during the incubation period of this study. Using a rat jejunum and ileum analysis, Spencer et al. [35] reported that transported hesperetin was mainly in the aglycone form. During transport through the jejunum or ileum, this flavonoid with only one substituted hydroxyl group on the B-ring is less susceptible to glucuronidation than flavonoids containing a 3′,4′-ortho-dihydroxy (or catechol) B-ring.

We have also reported that the $J_{ap\rightarrow bl}$ of paracellularly absorbed compounds such as gallic acid, catechins, chlorogenic acid, rosmarinic acid, and 3,4-dihydroxyphenylacetic acid (5 mM) is 0.10–0.30 nmol/min/mg protein [24,27,32,36]. The $J_{ap\rightarrow bl}$ of hesperidin at 0.5 mM (0.023±0.008 nmol/min/mg protein) was similar to that of the above paracellularly absorbed compounds, and therefore the similarity could be attributed to a common nonsaturable transport system (Fig. 2B) [24,27,32,36]. Furthermore, the transepithelial flux of hesperidin, both in the presence or absence of a proton gradient, was inversely correlated with TER (Fig. 2D). These findings indicate that hesperidin is also transported paracellularly. Kim et al. [37] have also reported that the mixture of hesperidin and its glycosides (formed by cyclodextrin glucanotransferase) crosses Caco-2 monolayers via the paracellular pathway. The addition of hesperetin (0.5 mM) or hesperidin (0.5 mM) to the Caco-2 cells did not significantly affect TER values. After the experiment in the presence of proton gradient, the TER value relative to the initial was 0.94 for hesperetin, and 0.93 for hesperidin, respectively. Similar results were observed in the absence of proton gradient.

The $J_{ap\rightarrow bl}$ of hesperetin (10.43±0.78 nmol/min/mg protein) was 5-fold higher than the $J_{bl\rightarrow ap}$ (2.02±0.24 nmol/min/mg protein) of hesperetin in the presence of a proton gradient (Fig. 2A), and apically loaded NaN_3 or CCCP inhibited the $J_{ap\rightarrow bl}$ by approximately 50%. In the absence of a proton gradient, the $J_{ap\rightarrow bl}$ and $J_{bl\rightarrow ap}$ of hesperetin (5.75±0.40 and 5.16±0.73 nmol/min/mg protein) were both about half the $J_{ap\rightarrow bl}$ of hesperetin in the presence of a proton gradient (Fig. 2A), and the $J_{ap\rightarrow bl}$ in the absence of a proton gradient was not inhibited by CCCP, as shown in the case of the $J_{ap\rightarrow bl}$ in the presence of a proton gradient (data not shown). These findings indicate that nearly half the $J_{ap\rightarrow bl}$ of hesperetin is proton coupled and that its flux is mediated by a polarized, energy-dependent transport system.

Quercetin 4′-β-glucoside is taken up into enterocytes across the brush border membrane by Na^+-driven active transport with SGLT-1 [19]. Therefore, we examined Na^+ dependency of hesperetin transport in the presence or absence of a proton gradient. The relative $J_{ap\rightarrow bl}$ of hesperetin under Na^+-free or containing conditions was almost the same both in the presence and absence of the proton gradient (in result section). These results clearly indicate that Na^+ did not affect the polarized, energy-dependent transport of hesperetin. We have also reported that 3,5-diprenyl-4-hydroxycinnamic acid (AC), an active ingredient of Brazilian propolis, mainly permeates Caco-2 cells by transcellular passive diffusion due to the ingredient's high lipophilicity [30]. The intracellular accumulation of hesperetin both in the presence and absence of proton gradient, which was shown in Table 2A, supported that hesperetin was transported via transcellular pathway. The transepithelial flux of hesperetin was constant irrespective of the TER (Fig. 2C). Furthermore, NaN_3 or CCCP did not affect about one-half of the $J_{ap\rightarrow bl}$ (Table 1A). Until now, it has been generally thought that flavonoids are absorbed by the liposomal membrane via transcellular passive diffusion [16,21]. However, we found that hesperetin is absorbed by a novel mechanism, involving proton-coupled, energy-dependent, polarized transport (Fig. 3).

Several proton-coupled transporters, like MCT and PEPT1, have been identified and partially characterized in small intestine [38,39]. However, the mechanisms under which these transporters involve are still complicated; for example, one of the tea flavonoids, (−)-epichatechin-3-gallate, is absorbed across the apical membrane of enterocytes via the MCT [29], although it is not a carboxylic acid.

To further characterize this proton-dependent transport of hesperetin, we examined the effects of inhibitors or substrates of the PEPT1 and MCT on hesperetin transport in Caco-2 cells. A substrate of PEPT1 (Gly-Sar [10 mM]) did not inhibit the directional transport of hesperetin in the presence of proton gradient (Table 1A), indicating that hesperetin is not transported by PEPT1. An inhibitor of MCT (phloretin [0.3 mM]) also did not inhibit, however, benzoic acid, substrate of MCT at 10 mM (but not 5 mM), slightly inhibited the hesperetin transport (Table 1A). It was reported that various subtypes of MCT, such as MCT1, MCT3, MCT4, MCT5 and MCT6, are expressed in Caco-2 cells [40]. MCTs are well known to transport short-chain fatty acid and carboxylic acid type of drugs [41]. The key components of substrates for MCTs are thought to be mono-anionic carboxylic acid group and nonpolar side chain or aromatic hydrophobic moiety [42]. However, hesperetin is not a carboxylic acid, and likely to be present in undissociated form under these experiments. Although the present results suggest that hesperetin seems to be transported via MCT, to some extent, however, it is still obscure that the transport system of hesperetin would be common with that of phenolic acids as previously demonstrated [26,27,30–33]. Further studies to characteristic this transport characteristics are required to fully assess the health effect of hesperetin.

In the absence of a proton gradient, both $J_{ap\rightarrow bl}$ and $J_{bl\rightarrow ap}$ of hesperetin were almost the same (5.75±0.40 and 5.16±0.73 nmol/min/mg protein) (Fig. 1A). However, $J_{bl\rightarrow ap}$ of hesperetin in the presence of a proton gradient was lower than $J_{bl\rightarrow ap}$ in the absence of a proton gradient (Fig. 1A). To clarify this observation, permeation and distribution of hesperetin were examined after loading it either apically or basolaterally with and without NaN_3 or CCCP (Tables 1B and 2B). Apically loaded NaN_3 and CCCP increased the $J_{bl\rightarrow ap}$ of hesperetin and caused the redistribution of basolaterally loaded hesperetin. The level of hesperetin increased markedly on the apical side and decreased markedly on the basolateral side. Basolaterally loaded NaN_3 exerted no effect on the distribution of hesperetin. Since NaN_3 and CCCP inhibit intracellular hesperetin uptake on the apical side, the hesperetin-specific active transport system is likely to be on the apical side. However, it is unknown whether hesperetin transport mechanisms differ on the apical and basolateral sides.

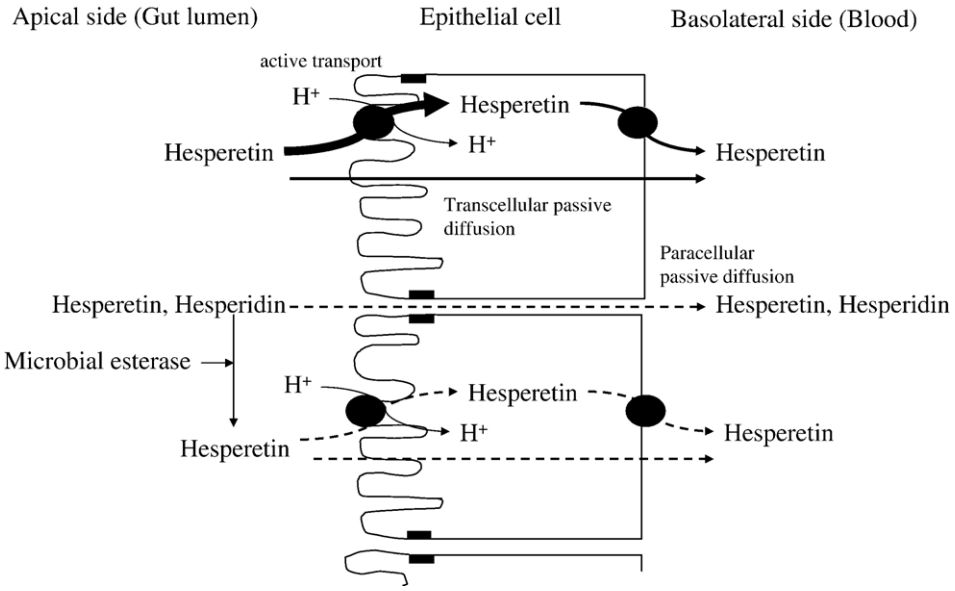


Fig. 3. Possible routes for absorption of the ingested hesperetin and hesperidin.

In *in vivo* studies, orally administered flavonoids such as rutin, hesperidin, naringin and narirutin, which contain rutinoses or neohesperidoses, are absorbed only in the distal part of intestine, after hydrolysis by intestinal enzymes of colonic microflora [14,15]. Izumi et al. [18] have reported that, in human, soy isoflavone aglycones containing genistein and daidzein were absorbed faster and in five times higher amounts than their glucosides. These findings support well our present data that hesperetin (aglycone) was absorbed more efficiently than hesperidin (glucoside).

In conclusion, the permeation rate of hesperetin was about 400 times that of hesperidin. Hesperidin is poorly transported via the paracellular route and is mainly absorbed from the intestine after hydrolysis by colonic microflora β-glucosidases. Hesperetin is absorbed mainly via proton-coupled active transport and transcellular passive diffusion. We have given novel insight to the absorption characteristics of hesperetin, that is proton-coupled and energy-dependent polarized transport.

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