Original paper

Paracellular Transport of Sulforaphane across Caco-2 Cell Monolayers

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The present study aimed to clarify the transport mechanism of sulforaphane (SFN), a potent phytochemical of broccoli, in Caco-2 cells. Transport study with an Ussing chamber system revealed the apparent permeability (P_{app}) of SFN in the apical-to-basolateral direction was much higher ($31.2 \pm 2.5 \times 10^{-6}$ cm/sec) than that of (-)-epigallocatechin-3-O-gallate ($0.54 \pm 0.12 \times 10^{-6}$ cm/sec). An efflux ratio analysis and several inhibition studies suggested SFN was passively transported across Caco-2 cell monolayers. Small amounts of glutathione conjugates of SFN in the basolateral side suggested that the transcellular pathway was, in part, involved in the transport of SFN. However, results showing that SFN transport was attenuated by sodium butyrate, a tight junction (TJ) closer, and competed with fluorescein, a selective marker of paracellular transport, clearly indicated that SFN was mainly transported through the TJ-controlled paracellular pathway.

Keywords: sulforaphane, transport, paracellular pathway, Caco-2, tight junction

Introduction

Epidemiological studies have demonstrated that a higher consumption of cruciferous vegetables, including broccoli, cabbage and kale, is likely to contribute to the lowered risk of various cancers and lifestyle-related chronic diseases (Higdon *et al.*, 2007; Yamaji *et al.*, 2008; Fahey *et al.*, 2012). As with other kinds of vegetables, cruciferous vegetables are also excellent sources of nutrients and phytochemicals including carotenoids, polyphenols, and sulfur-containing compounds. They characteristically contain a variety of glucosinolates, stable precursors of isothiocyanates, which show diverse physiological actions by interacting with proteins due to their electrophilic properties (Dinkova-Kostova and Kostov, 2012). One well-known isothiocyanate is sulforaphane [SFN; 1-isothiocyanato-4-(methylsulfinyl)-butane], which was identified as a potent inducer of cytoprotective genes including phase 2 detoxification enzymes and antioxidant proteins (Zhang *et al.*, 1992; Fahey *et al.*, 1997). SFN has also been shown to promote the detoxification of exo/endogenous toxicants and carcinogens,

such as aflatoxin (Kensler *et al.*, 2005), acetaldehyde (Ushida and Talalay, 2013), methylmercury (Toyama *et al.*, 2011) and acrolein (Egner *et al.*, 2014), as well as moderate oxidative radicals (Gaona-Gaona *et al.*, 2011). Numerous animal studies have demonstrated that SFN showed some preventive effects against cancer (Talalay *et al.*, 1995; Kensler *et al.*, 2013), liver failure (Sayed *et al.*, 2014), type 2 diabetes-induced cardiomyopathy (Zhang *et al.*, 2014), cerebral nerve diseases (Shirai *et al.*, 2012, 2015), arthritis (Kong *et al.*, 2010), asthma (Park *et al.*, 2012), and macular degeneration (Gao and Talalay, 2004). Furthermore, clinical studies suggested the possible efficacy of SFN intake in the prevention and/or improvement of skin erythema (Talalay *et al.*, 2007), autism (Singh *et al.*, 2014), insulin resistance (Bahadoran *et al.*, 2012), *Helicobacter pylori*-infection (Yanaka *et al.*, 2009) and liver abnormality (Kikuchi *et al.*, 2015). Therefore, there is a growing demand for the development of SFN-based nutraceutical products.

Knowledge of absorption, distribution, metabolism, and excretion (so called ADME) allows us to predict the bioavailability

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and *in vivo* bioactivity of target compounds including phytochemicals. Clinical studies on the absorption of SFN demonstrated that the intake of SFN showed much higher SFN absorption compared to the intake of its precursor glucoraphanin (Egner *et al.*, 2011; Cramer *et al.*, 2012), since glucoraphanin must be converted to SFN by myrosinase-producing gut microflora prior to intestinal absorption. ADME studies also revealed that absorbed SFN was metabolized via the mercapturic acid pathway, distributed at organs such as prostate, kidney, heart, and brain, and excreted within 10 h (Clarke *et al.*, 2011; Bricker *et al.*, 2014). However, to date, the intestinal transport mechanism of SFN remains unclear. Thus, in the present study, we aimed to clarify the transport pathway(s) of SFN using Caco-2 cell transport experiments.

Materials and Methods

Materials SFN (as *R,S*-sulforaphane) was purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada). Phloretin was from Wako Pure Chemical Industries (Osaka, Japan). Fluorescein sodium salt, sodium butyrate, and (-)-epigallocatechin-3-*O*-gallate (EGCg) were from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from GIBCO Life Technologies (Grand Island, NY). Wortmannin was from ENZO Life Sciences (East Farmingdale, NY). *R*-Iberin (IBN; 1-isothiocyanato-3-(methylsulfonyl)-propane) was purchased from LKT Laboratories, Inc. (St. Paul, MN). Glutathione conjugate of SFN was from Santa Cruz Biotechnology (Dallas, TX). All other chemicals were of analytical reagent grade and were used without further purification.

Cell culture Caco-2 cells were cultured according to the method in our previous report (Takeda *et al.*, 2013). Caco-2 cells were cultured in DMEM supplemented with 10% FBS, 1% nonessential amino acids (MP Biomedicals, Irvine, CA), 2 mM L-glutamine (Nacalai Tesque, Kyoto, Japan), 100 U/mL penicillin (Meiji Seika Co., Tokyo, Japan), 100 µg/mL streptomycin (Nacalai Tesque), and 1.7 µM insulin (Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO₂ in air. The monolayers became confluent 4–5 days after seeding at a density of 1.0×10^6 cells per 100 mm dish, and the cells were passaged at a split ratio of 4–8 by treatment with 0.25% trypsin and 0.02% di-sodium salt of ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS: Cosmo Bio Co., Tokyo, Japan). Caco-2 cells used in this study were between passages 50 and 60. For the transport study, the cells seeded at a density of 4.0×10^5 cells/mL were grown in BD Falcon cell culture inserts (polyethylene terephthalate membrane, 0.9 cm², 1.0 µm pore size; BD Biosciences, Bedford, MA) with a type I collagen-coated membrane (collagen gel culturing kit, Cellmatrix type I-A; Nitta Gelatin, Osaka, Japan). The cells were cultured using a BD BioCoat™ intestinal epithelium differentiation kit (BD Biosciences), by which monolayers were formed after 6 days. The medium was changed every day. The integrity of the monolayers was evaluated by

measuring transepithelial electrical resistance (TEER) using a Millicell®ERS-2 (EMD Millipore, Darmstadt, Germany). Monolayers with TEER values > 100 Ω cm² were used for transport experiments.

Transport study of SFN across Caco-2 cell monolayers A transport study was performed with Caco-2 cell monolayers in an Ussing chamber system (Model U-2500; Warner Instrument Corporation, Hamden, CT) as described previously (Zhu *et al.*, 2008). Caco-2 cell monolayers grown in the transwell inserts were gently rinsed with Hank's balanced salt solution (HBSS, pH 6.0) before the experiments. An aliquot (6.0 mL) of HBSS buffer (pH 6.0, adjusted with 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES)) was added to the apical side of the chamber, and 6.0 mL of HBSS buffer (pH 7.4, adjusted with 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)) were added to the basolateral side. After equilibration for 15 min at 37°C, the transport studies were started by replacing the HBSS buffer with 6.0 mL of SFN solution in the presence or absence of inhibitors (1 and 100 µM wortmannin, a phosphoinositide 3-kinase (PI-3K) inhibitor; 100 and 300 µM phloretin, a monocarboxylic acid transporter (MCT) inhibitor) (Basquin *et al.*, 2013; Halestrap & Meredith, 2004); cells were treated for 30 min prior to the transport study. Cells were treated with sodium butyrate (2 mM), a tight junction closer (Peng *et al.*, 2009), for 24 h before the study. During the transport experiments, the solutions in both sides were maintained at 37°C and bubbled continuously with a mixture of O₂:CO₂ (95:5) through air vents in the chamber. At time intervals up to 60 min, a sample (0.21 mL) was drawn from the basolateral side and replaced with the same volume of fresh buffer. For the transport experiments in the reverse direction from basolateral to apical side, 6.0 mL of HBSS buffer (pH 7.4) in the basolateral side were replaced with 6.0 mL of SFN solution (pH 7.4) to start the transport experiments, and a sample (0.21 mL) was taken from the apical side and replaced with fresh buffer. The solutions collected in the time course of transport studies were subjected to liquid chromatography electrospray ionization time-of-flight-mass spectrometry (LC-ESI-TOF-MS) analysis for the quantification of transported SFN as described below. At the end of experiments, the viability of the Caco-2 cell monolayer was confirmed by no significant decrease of TEER value compared to that at the beginning.

The apparent permeability coefficient (P_{app}) was calculated from the following equation:

$$P_{app} \text{ (cm/sec)} = (dC/dt) \times V / (A \times C_0) \quad \text{.....Eq. 1}$$

where dC/dt is the change in concentration at the basolateral side over time (µM/sec), V is the volume of solution in the basolateral compartment (6.0 mL), A is the surface area of the membrane (0.2826 cm²), and C_0 is the initial concentration at the apical side (µM).

LC-ESI-TOF-MS analysis The concentration of SFN in the

transported solution was determined using LC-ESI-TOF-MS. Briefly, an aliquot (20 μ L) of sample from the transport experiment was applied to the system. LC separation was performed with an Agilent 1200 series (Agilent, Waldbronn, Germany) on a Cosmosil 5C₁₈-AR-II column ($\varnothing$ 2.0 mm $\times$ 150 mm, Nacalai Tesque) at 40°C. The mobile phase consisted of 0.1% formic acid (FA) (solvent A) and acetonitrile with 0.1% FA (solvent B) using a 20 min-linear gradient from 0% to 100% of solvent B at a flow rate of 0.2 mL/min. MS analysis was performed using micrOTOF II (Bruker Daltonics, Bremen, Germany). The amount of SFN (m/z 178.0355) was analyzed in positive ion mode. The MS conditions were as follows: drying gas, N₂; flow rate, 8.0 L/min; drying gas temperature, 200°C; drying gas pressure, 1.6 bar; and capillary voltage 4,500 V. The calibration solution of 10 mM sodium formate in 50% acetonitrile was injected at the beginning of the run. The data were analyzed and acquired using the Bruker Data Analysis 3.2 software.

Fluorescein transport assay Fluorescein transport experiments were performed using a series of fluorescein concentrations (10, 30, 50, 100 μ M) in the absence or presence of SFN (50 or 100 μ M) (pH 7.4 at both apical and basolateral sides). At time points of 5, 10, 15, 20, 25 and 30 min for the transport experiment, the amount of transported fluorescein was determined with a fluorescence spectrophotometer (Wallac ARVO SX 1420 Multilabel Counter; Perkin Elmer Life Sciences, Tokyo, Japan) at an excitation wavelength of 490 nm and emission wavelength of 514 nm. Fluorescein flux expressed in nmol per min per cm² was determined and subjected to Lineweaver–Burk plot analysis.

Statistical analysis Data are expressed as the mean $\pm$ SEM. Differences between two groups were analyzed by an unpaired Student's *t*-test. Differences among three groups were analyzed by a one-way ANOVA. Differences at $p < 0.05$ were considered to be statistically significant. All analyses were performed using Graph Pad Prism version 5.0 for Windows (Graph Pad Software, La Jolla, CA).

Results

Transport of SFN across Caco-2 cell monolayers Transport of SFN across Caco-2 cell monolayers was primarily examined by measuring the time-course change in SFN transport from the apical to basolateral side. As shown in Fig. 1, the transport of SFN to the basolateral side was successfully detected by the present LC-ESI-TOF-MS analysis. The estimated limit of detection for SFN under the MS conditions was ca. 1.0 pmol/injection (data not shown). The amount of transported SFN across Caco-2 monolayers increased in a time-dependent manner up to 60 min, and the calculated P_{app} was $31.2 \pm 2.5 \times 10^{-6}$ cm/sec. Treatment with 100 μ M SFN in the present transport study did not result in any toxicity against Caco-2 cells. Moreover, no significant difference in TEER value between before ($344.9 \pm 26.1 \Omega \text{ cm}^2$) and after 60 min-SFN transport study ($354.9 \pm 21.6 \Omega \text{ cm}^2$) ensured that 100 μ M

Fig. 1. Stacked extracted ion chromatograms (EICs) of SFN measured by ESI-TOF-MS at 15, 30, 45, and 60 min in the transport study using Caco-2 cell monolayers. The time-course transport of SFN (100 μ M) was measured from the apical to basolateral side of Caco-2 cell monolayers.

Table 1. Summary of P_{app} values

Compound	P_{app} ($\times 10^{-6}$ cm/sec)
SFN	31.2 ± 2.5
IBN	28.2 ± 1.0
EGCg	0.54 ± 0.12
Gly-Sar	21.8 ± 1.8^a

a: From the report of Takeda *et al.* (2013)

SFN had no toxic effects.

IBN, an analogous compound to SFN but with one carbon shorter alkyl chain, was also employed in the Caco-2 transport experiments to reveal SFN transport characteristics. As summarized in Table 1, P_{app} value of IBN ($28.2 \pm 1.0 \times 10^{-6}$ cm/sec) was comparable to that of SFN, suggesting that molecular size or alkyl chain length did not likely affect SFN transport ability. Considering the transport abilities of EGCg ($0.54 \pm 0.12 \times 10^{-6}$ cm/sec) and the peptide-transporter model, Gly-Sar ($21.8 \pm 1.8 \times 10^{-6}$ cm/sec; Takeda *et al.*, 2013), SFN must be categorized as one of the favorable natural penetrants.

Directional characteristics of transepithelial transport of SFN To assess the directional characteristics of SFN transport across Caco-2 cell monolayers, reverse transport of SFN from basolateral to apical side was investigated. As shown in Fig. 2, SFN showed similar transport behavior irrespective of direction. P_{app} value of SFN from basolateral to apical side was $51.1 \pm 13.1 \times 10^{-6}$ cm/sec, and the efflux ratio, which can be calculated by the ratio of P_{app} from basolateral to apical divided by P_{app} from apical to basolateral, was 1.6. According to the criteria of efflux ratio proposed by Farrell *et al.* (2012), a passive transport pathway might be involved in the SFN transport across Caco-2 cell monolayers.

Fig. 2. Changes in the concentration of transported SFN across Caco-2 cell monolayers from the apical to basolateral side (A-B), and from the basolateral to apical side (B-A). Results are expressed as the mean $\pm$ SEM ($n = 3$).

Fig. 3. Effects of phloretin (A) and wortmannin (B) on SFN transport across Caco-2 cell monolayers. Caco-2 cell monolayers were treated with phloretin (100 and 300 μ M) as MCT inhibitor, or wortmannin (1 and 100 μ M) as endocytosis inhibitor for 30 min prior to the transport study. Transport of SFN from apical to basolateral side was measured in the absence or presence of the inhibitors. Results are expressed as the mean $\pm$ SEM ($n = 3$). *N.S.*, no significant difference (one-way ANOVA).

Transport pathways of SFN across Caco-2 cell monolayers

To investigate the transport pathway of SFN, possible carrier-mediated transporter pathways were primarily examined, in light of the reported interaction of the monocarboxylic transporter (MCT) with reactive isothiocyanate groups (Poole and Halestrap, 1997;

Fig. 4. Effect of sodium butyrate on TEER value of Caco-2 cell monolayers (A) and SFN transport (B). Caco-2 cell were treated with 2 mM sodium butyrate, a tight junction closer, 24 h before the transport study of SFN (100 μ M). Transport of SFN from apical to basolateral side of Caco-2 cell monolayers was measured. Results are expressed as the mean $\pm$ SEM ($n = 3$). Statistical differences between the two groups were analyzed by a Student's *t*-test.

Wilson *et al.*, 2009). As shown in Fig. 3A, phloretin (100 and 300 μ M), an MCT inhibitor (Halestrap and Meredith, 2004), did not affect the SFN transport. In addition, wortmannin, which is an inhibitor of endocytosis by PI-3K inhibition (Basquin *et al.*, 2013), did not affect the SFN transport. Collectively, these findings suggested that carrier-mediated transport would be excluded as a possible SFN transport pathway.

The involvement of the paracellular pathway in SFN transport across Caco-2 cell monolayers was then investigated. As shown in Fig. 4, 2 mM sodium butyrate as a tight junction closer (Peng *et al.*, 2009) significantly ($p < 0.05$) decreased the P_{app} of SFN, together with the enhanced TEER, suggesting that SFN may be transported through the paracellular pathway. The results that the transport of fluorescein, an indicator of paracellular transport (Rosenthal *et al.*, 2010), was competitively inhibited by SFN (Fig. 5) also indicated

Fig. 5. Lineweaver-Burk plots of fluorescein transport across Caco-2 cell monolayers in the presence (50 or 100 μM) or absence of SFN. Fluorescein flux was determined at a concentration range from 10 to 100 μM of fluorescein. Results are expressed as the mean $\pm$ SEM ($n = 3 - 4$).

Fig. 6. EIC chromatograms of transported SFN and metabolites after 60-min transport study. SFN (100 μM) was subjected to the transport study from the apical to basolateral side of Caco-2 cell monolayers for 60 min. HBSS buffer was then collected to analyze SFN and mercapturic acid metabolites such as *N*-acetylcysteine (NAC)-, cysteinylglycine (Cys-Gly)-, glutathione (GSH)-, and cysteine (Cys)-SFN.

the possibility of paracellular transport of SFN across Caco-2 cell monolayers.

Mercapturic acid metabolites of SFN in basolateral side of Caco-2 cell monolayers To examine whether SFN was metabolized by the mercapturic acid pathway, possible metabolites of SFN such as glutathione (GSH)-, *N*-acetylcysteine (NAC)-,

cysteinylglycine (Cys-Gly)-, and cysteine (Cys)-conjugates in the basolateral side were analyzed after 60 min-Caco-2 transport experiments by LC-ESI-TOF-MS. Figure 6 shows the extracted ion chromatograms (EICs) for SFN and the metabolites in the basolateral side solution. As a result, no detectable peaks were observed in the EICs monitored at m/z 341.0658 (NAC-SFN), 356.0767 (Cys-Gly-SFN), and 299.0552 (Cys-SFN), except GSH-SFN (m/z 485.1193), a primary metabolite in the mercapturic acid pathway. However, the amount of observed GSH-SFN metabolite in the basolateral side was estimated to be ca. 1/60-fold lower than that of transported SFN. No metabolites were detected in the apical side after 60 min-Caco-2 transport experiments.

Discussion

SFN, a potent phytochemical in broccoli, is readily absorbed, distributed, metabolized, and then excreted into the urine within ca. 10 h of consumption (Clarke *et al.*, 2011; Bricker *et al.*, 2014). However, the molecular mechanism of SFN transport across the intestinal epithelium requires clarification. The racemic SFN used in the present study was presumed to be absorbed from the intestine as previously reported for naturally occurring *R*-SFN (Hanlon *et al.*, 2008; Cramer *et al.*, 2012; Egner *et al.*, 2011). The present Caco-2 transport study with the Ussing chamber system demonstrated that SFN was time-dependently transported across Caco-2 cell monolayers from apical to basolateral side with a high P_{app} value of $31.2 \pm 2.5 \times 10^{-6}$ cm/sec. This value was comparable to that for IBN, and was much higher than that for EGCg (P_{app} : $0.54 \pm 0.12 \times 10^{-6}$ cm/sec), which is supported by the reported low bioavailability of EGCg (Miyazawa, 2000). An efflux ratio analysis and several studies with an MCT inhibitor and an endocytosis inhibitor indicated that the SFN transport across Caco-2 cell monolayers was passive, not carrier-mediated active transport.

A major finding of the present study was the involvement of the paracellular pathway in the passive transport of SFN. Attenuation of SFN transport by sodium butyrate and competitive inhibition of fluorescein transport by SFN (Figs. 4 and 5) also revealed the paracellular pathway as the main SFN transport in Caco-2 cells. The paracellular pathway, which is formed by tight junction proteins composed of three integral protein families, claudins, occludins, and junction adhesion molecules (Tsukita *et al.*, 2008), is regarded as an important transport route for some drug molecules and nutrients. This pathway has been an attractive target for increasing the bioavailability of active compounds in pharma- and nutraceuticals because of their ability to be modulated by a wide variety of compounds in foods identified as openers and closers of tight junction proteins (Suzuki and Hara, 2011; Yeh *et al.*, 2011; Park *et al.*, 2015). Thus, our findings provide useful information for developing nutraceuticals based on SFN and other isothiocyanates from cruciferous vegetables. Future studies will examine the paracellular transport of SFN using an *ex vivo* experimental system with intestinal membranes isolated from rats

and further *in vivo* animal experiments.

Passive transcellular transport of SFN was predicted due to its high lipophilicity [$\log P$ (octanol/water) = 0.72] (Cooper *et al.*, 1997; Winiwater *et al.*, 1998) and uptake in mammalian cells (Zhang and Collaway, 2002; Fahey *et al.*, 2002); a previous study demonstrated the presence of GSH-SFN, but not other metabolites, in the basolateral side of a human jejunum *in vivo* model (Petri *et al.*, 2003). This is in accordance with our data (Fig. 6), suggesting that SFN might be partially transported through the transcellular pathway. Metabolites of SFN in the mercapturic acid pathway such as Cys- and NAC-SFN were not observed in the basolateral side in the current study, although they have been found in the mouse small intestine after single gavage of SFN (Clarke *et al.*, 2011). This can be potentially explained by the substrate specificity of multidrug resistance associated protein 1 (MRP-1), a transporter expressed in intestine, liver and so on, and which is involved in the basolateral efflux of compounds, especially GSH- and glucuronate-conjugates (Cole and Deeley, 2006). From past experiences, moreover, we acknowledge that transport rates of compounds across Caco-2 cell monolayers tend to be faster than those across intestinal membranes isolated from animals (Takeda *et al.*, 2013; Matsui *et al.*, 2006), which might influence the metabolism of SFN in enterocytes in this study. Thus, we will clarify the mechanism of transcellular transport and the metabolism of SFN in future *ex vivo* and *in vivo* animal studies in addition to those already described.

In conclusion, the present study provides the first evidence that SFN, a potent phytochemical in broccoli and cruciferous vegetables, can be passively transported across Caco-2 cell monolayers, mainly through the paracellular pathway as well as the previously characterized transcellular pathways. This finding would contribute to the development of nutraceuticals containing SFN and related phytochemicals exhibiting high absorption efficiency.

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