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Substrate Specificity of the Nateglinide/H^+ Cotransport System for Phenolic Acids

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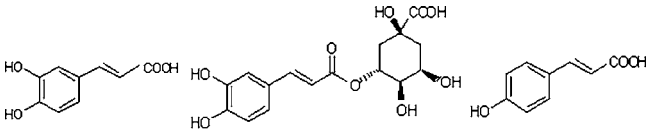
In clinical, patients usually take many kinds of drugs at the same time. Thus, drug−drug interactions involving transporters can often directly affect the therapeutic safety and efficacy of many important drugs. However, there have been few studies on food−drug interactions involving transporters. Dietary polyphenols have been widely assumed to be beneficial for human health. Polyphenols are found ubiquitously, and they are commercially prepared and used as functional foods. We have reported that ferulic acid, which is one of the most well-known polyphenols and is used as a functional food, affected the transport of nateglinide, an antidiabetic drug, by Caco-2 cells. In this study, we investigated the effects of other polyphenols on the nateglinide/H^+ transport system. We report here that caffeic acid and *p*-coumaric acid have a different inhibitory manner on the uptake of nateglinide. The results of this study are useful to identify the substrate specificity of the nateglinide/H^+ cotransporter.

KEYWORDS: Nateglinide; fluorescein; caffeic acid; phenolic acid; transporter

INTRODUCTION

(−)-*N*-(*trans*-4-Isopropylcyclohexanecarbonyl)-D-phenylalanine (nateglinide) is a novel oral hypoglycemic agent possessing a carbonyl group and a peptide bond in its structure. Nateglinide is a non-sulfonylurea antidiabetic agent that stimulates insulin secretion via the same mechanism as that by which insulin secretion is stimulated by sulfonylureas, although nateglinide induces a more rapid and briefer decrease in blood glucose level than do sulfonylureas (*1*−*3*). The unique feature of nateglinide is rapid intestinal absorption and renal elimination. Since nateglinide is an anionic compound with a pK_a of 3.1, it exists predominantly in ionized form at the intestinal physiological pH of 6.5. Moreover, its chloroform/water partition coefficient has been reported to be 0.2 at pH 6.8, indicating that it is hardly lipophilic (*4*). These physicochemical features are incompatible with rapid absorption by passive diffusion, suggesting that nateglinide is absorbed via a specific transport system(s) in the intestine. Recently, we have reported that nateglinide shares an inwardly H^+-driven transporter with fluorescein by Caco-2 cells (*5*, *6*).

We have also reported that ferulic acid, which is one of the most well-known polyphenols, affects the transport of nateglinide. Polyphenols are the most abundant antioxidants in our daily food and play a role in prevention of diseases associated with oxidative stress (*7*−*9*). Dietary polyphenols are thought to be profitable to human health by exerting various biological effects such as metal chelation, free radical scavenging, alternation of signal transduction pathways, and modulation of enzymatic activity. Epidemiological studies have already shown the relationships between consumption of polyphenol-rich foods and the prevention of diseases such as various cancers, coronary heart disease, and osteoporosis (*10*−*12*). For evaluation of the role of dietary polyphenols in the prevention of diseases, it is important to have knowledge of the bioavailability. Polyphenols are classified into flavonoids, phenolic acids, and less common stilbenes and lignans. Many studies have focused on the absorption and metabolism of flavonoids (*13*−*15*). However, there have not been many studies on phenolic acids such as ferulic acid and caffeic acid (*16*−*19*), despite their high contents in fruits, vegetables, and grains (*20*). Caffeic acid is one of the major representative of the hydroxycinnamic acids and exists in foods (**Figure 1**; **Table 1**). Coffee is the main source of caffeic acid in the diet. Caffeic acid exhibits antioxidant activities and has a preventative effect on oral cancer (*21*−*23*). We have already reported that ferulic acid, which is also a famous hydroxycinnamic acid as caffeic acid, is transported across human intestinal Caco-2 cells by the nateglinide/H^+ cotransport system (*24*). However, it has been reported that caffeic acid does not inhibit fluorescein transport (*25*). It is possible that the transport system of caffeic acid for the nateglinide/H^+ cotransport system is partly different from that of ferulic acid even though its structure is similar to the structures of other phenolic acids. Konishi et al. have reported that ferulic acid



Caffeic acid **Chlorogenic acid** **p-Coumaric acid**

Figure 1. Chemical structures of caffeic acid, chlorogenic acid, and *p*-coumaric acid.

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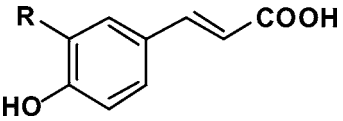
Table 1. Structures of Phenolic Acids and Their Inhibitory Effects on Uptake of Nateglinide by Caco-2 Cells



compound	R	manner of inhibition	K_i
caffeic acid	OH	noncompetitive	5.1
p-coumaric acid	H	competitive	2.1
ferulic acid[a]	OCH_3	competitive	1.2

[a] Reference *24*.

and caffeic acid are transported by the MCT-like transporter in intestinal Caco-2 cells (*18, 19*). However, there have not been reports of these substrates in detail. This study was designed to determine the substrate specificity of the nateglinide/H^+ cotransport system for phenolic acids, including caffeic acid.

MATERIALS AND METHODS

Chemicals. Caffeic acid, *p*-coumaric acid, and ferulic acid were purchased from Sigma (St. Louis, MO). Nateglinide was kindly donated by Yamanouchi (Tokyo, Japan). Fluorescein was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents were of the highest grade available and used without further purification.

Cell Culture. Caco-2 cells obtained from American Type Culture Collection (Rockville, MD) were maintained in plastic culture flasks (Falcon, Becton Dickinson and Co., Lincoln Park, NJ) as described previously (*26*). The medium used for growth of Caco-2 cells was Dulbecco's modified Eagle's medium (Gibco, Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum (ICN Biomedicals, Inc., Aurora, OH), 1% nonessential amino acids (Gibco), 4 mM glutamine (Gibco), and 100 IU/mL penicillin−100 μg/mL streptomycin. The monolayer cultures were grown in an atmosphere of 5% CO_2−95% O_2 at 37 °C. Cells reached confluency after 4−6 days in culture. The cells were harvested with 0.25 mM trypsin and 0.2% EDTA (5 min at 37 °C), resuspended, and seeded into a new flask. Cells between the 48th and 56th passages were used in this study. For the uptake study, Caco-2 cells were seeded at a cell density of (1−3) × 10^5 cells/cm^2 on 12-well plastic plates (Corning Coster Corp., Cambridge, MA). The cell monolayers were fed a flesh growth medium every 2 days and were then used at 4−6 days for the uptake experiments.

Uptake Studies in Caco-2 Cell Monolayers. The uptake experiment was performed as described previously (*27*). The uptake of substrates was measured using monolayer cultures grown in 12-well plastic plates. The composition of incubation medium was as follows: 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.3 mM KH_2PO_4, 0.4 mM NaH_2PO_4, 4.2 mM $NaHCO_3$, 25 mM D-glucose, and 10 mM MES (pH 5.5). After removal of the growth medium, cells were preincubated at 37 °C for 10 min with 1 mL of incubation medium. After removal of the medium, 0.5 mL of incubation medium containing substrates was added. The monolayers were incubated for an indicated time at 37 °C. Each cell monolayer was washed rapidly twice with 1 mL of an ice-cold incubation medium at the end of the incubation period. The cells were suspended in 0.4 mL of an extraction solution [1 N H_3PO_4/methanol (50/50)] for 1 h at room temperature. The extraction solution was used for the determination of substrate concentration after centrifugation at 12000*g* for 10 min.

Analytical Procedures. Substrates were determined using an HPLC system. Nateglinide was determined using an HPLC system equipped with a JASCO 880-PU pump 870-UV UV/vis detector described previously (*28*). The column was a CERI L-column ODS (4.6 mm i.d. × 150 mm). In the assay for nateglinide, a mobile phase containing acetonitrile/50 mM H_3PO_4, pH 2.5 (45/55), was used. The column temperature and flow rate were 55 °C and 0.7 mL/min, respectively. The wavelength for detection of nateglinide was 210 nm. The lower limit of quantitation for nateglinide was 250 pmol/mL, respectively. The reproducibility was good without requiring any sample pretreatment. The retention time for nateglinide was 8.5 min. The measurement of fluorescein was carried out in a spectrofluorometer (Hitachi 950-60) with an excitation wavelength of 490 nm and emission wavelength of 525 nm. Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard (*29*). Analysis of variance (ANOVA) and Student's *t*-test were used for the statistical analysis, and a value $P < 0.05$ was considered significant. Nonlinear regression analysis and least-squares fitting for the Eadie−Hofstee plot of substrate uptake were performed by using Origin (version 6.1J).

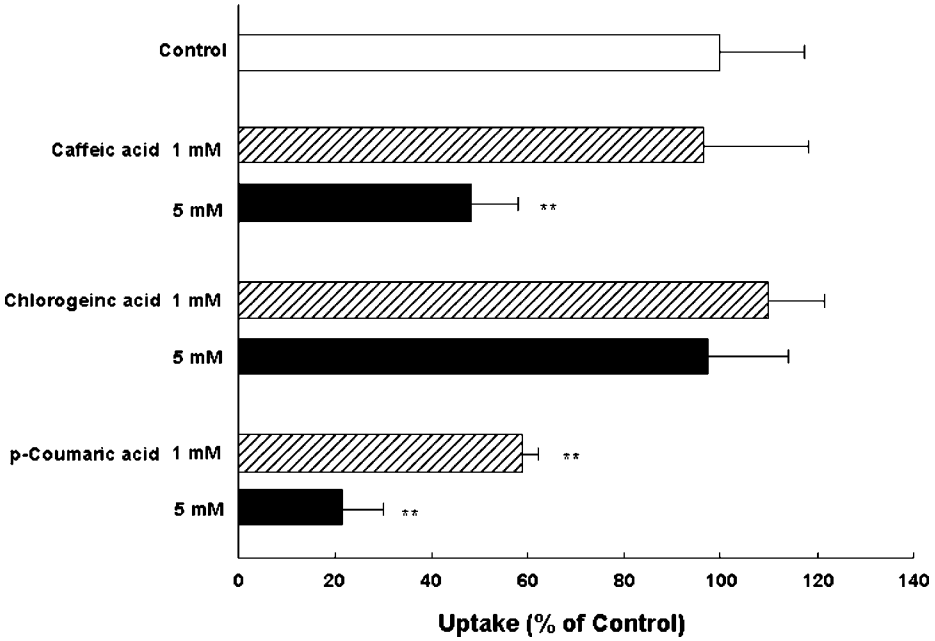


Figure 2. Inhibitory effects of phenolic acids on nateglinide uptake by Caco-2 cells. Uptake of 50 μM nateglinide was measured over a period of 5 min in the presence of an inwardly directed H^+ gradient. Each column represents the mean with SD of three to five measurements. The control value of the uptake of nateglinide was 4.24 ± 0.48 nmol (mg of protein)$^{-1}$ (5 min)$^{-1}$. **, $P < 0.01$ significantly different from the control.

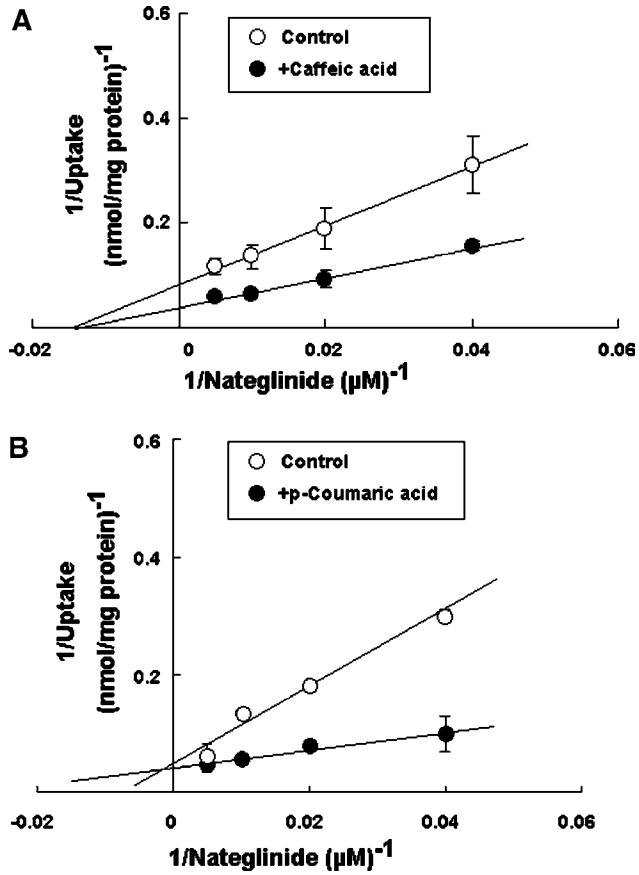


Figure 3. Lineweaver–Burk plot of H^+-driven nateglinide uptake by Caco-2 cells. Uptake of nateglinide was measured in the presence or absence of 5 mM caffeic acid (A) and 5 mM *p*-coumaric acid (B). Each point represents the mean with SD of three to five measurements.

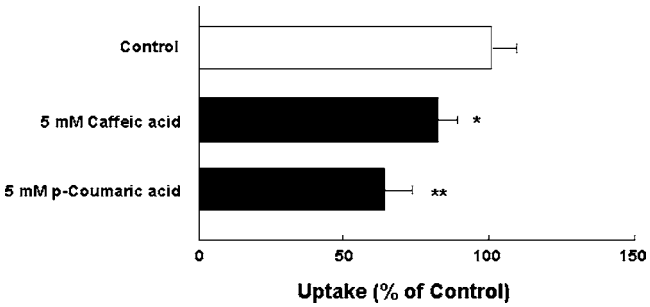


Figure 4. Inhibitory effects of caffeic acid and *p*-coumaric acid on the uptake of fluorescein. Uptake of 50 μM fluorescein was measured over a period of 5 min in the presence of an inwardly directed H^+ gradient. Each column represents the mean with SD of three to five measurements. The control value of the uptake of fluorescein was 2.41 ± 0.14 nmol (mg of protein)$^{-1}$ (5 min)$^{-1}$. *, $P < 0.05$; **, $P < 0.01$ significantly different from the control.

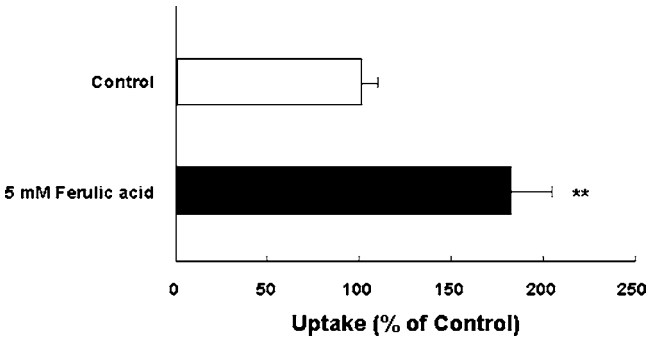


Figure 5. Trans stimulation of fluorescein uptake by ferulic acid. Incubation conditions were identical to those described in the legend to Figure 4. Each column represents the mean with SD of three to five measurements. The control value of the uptake of fluorescein was 2.41 ± 0.14 nmol (mg of protein)$^{-1}$ (5 min)$^{-1}$. **, $P < 0.01$ significantly different from the control.

RESULTS

Inhibitory Effects of Polyphenols on the Uptake of Nateglinide. To characterize the carrier that is responsible for the uptake of nateglinide by Caco-2 cells, we examined the inhibitory effects of polyphenols on the uptake of nateglinide (**Figure 2**). Caffeic acid and *p*-coumaric acid significantly inhibited the uptake of nateglinide. The inhibitory effect of caffeic acid on the uptake of nateglinide was less than that of *p*-coumaric acid. Chologenic acid, which does not possess a carboxyl group, did not have an effect on the uptake of nateglinide.

Kinetic Analysis of the Uptake of Nateglinide in the Presence of Caffeic Acid or *p*-Coumaric Acid. We have reported that ferulic acid transport occurs via an H^+-dependent system, which is identical to the nateglinide/fluorescein transport system (*24*). We investigated the kinetics of inhibitory effects of caffeic acid and *p*-coumaric acid on the uptake of nateglinide. **Figure 3** shows the effects of caffeic acid and *p*-coumaric acid on the uptake of nateglinide in terms of a Lineweaver−Burk plot. *p*-Coumaric acid inhibited the uptake of nateglinide competitively, and its apparent K_i value was 2.0 mM. On the other hand, caffeic acid inhibited the uptake of nateglinide in a noncompetitive manner.

Inhibitory Effects of Phenolic Acids on the Uptake of Fluorescein. Konishi et al. reported that phenolic acids had inhibitory effects on the permeation of fluorescein (*25*). Since the transcellular transport involves the uptake of compounds across apical membranes into cells and efflux across basolateral membranes, there are some differences between the transcellular transport and uptake experiments. On the other hand, the Caco-2 cells were grown in wells, and the basolateral transporter made a minor contribution to the efflux of compounds (*30*). To evaluate the inhibitory effects of phenolic acids on the uptake of fluorescein via apical-localized fluorescein transporter, effects of these substances on the uptake of fluorescein were examined. Caffeic acid and *p*-coumaric acid significantly inhibited the uptake of fluorescein (**Figure 4**). Although ferulic acid significantly inhibited the uptake of nateglinide (*24*), ferulic acid increased the uptake of fluorescein (**Figure 5**).

DISCUSSION

Transporter-mediated drug−drug interactions involving drugs having a narrow therapeutic range might have serious adverse consequences. However, there have been few studies about food−drug interactions involving transporters. Hydroxycinnamic acids are one of the groups of polyphenols that exist in the diet, mostly in whole grains, vegetables, fruits, and beverages (especially in wine) (*22*, *31*, *32*). Polyphenols have received much attention recently due to their strong antioxidant activity, which has been associated with beneficial effects of polyphenol-rich foods on human health (*33*).

We have recently shown that ferulic acid, which is widely used as a functional food, affects the transport of the clinical agent nateglinide, and we have also recently reported that the intestinal fluorescein transporter contributes to the uptake of nateglinide (*5*, *24*). These findings suggest that nateglinide interacts with not only coadministered drugs but also foods, including functional foods. It is important to evaluate food−

drug interactions involving the intestinal nateglinide/fluorescein transport system. In the present study, uptake experiments using well-grown Caco-2 cells were carried out in order to evaluate the function of the apical-localized nateglinide transporter.

Caffeic acid can easily form a resonance-stabilized phenoxyl radical, which is charged with potent antioxidant activity. Caffeic acid has been shown to have a potent antioxidant effect and a preventative effect on oral cancer (*21*−*23*). We have many opportunities to ingest functional foods including phenolic acids. We therefore focused on the caffeic acid. We found that caffeic acid, which is mostly ingested from coffee, affects the transport of clinical agents.

In the first part of this study, the inhibitory effects of caffeic acid, chlorogenic acid, and *p*-coumaric acid on the uptake of nateglinide were investigated. Caffeic acid inhibited H^+-dependent nateglinide uptake noncompetitively. On the other hand, chlorogenic acid did not affect the uptake of nateglinide. These results suggest that a monocarboxylate group contributes to the inhibitory effects of phenolic acids on the nateglinide/H^+ transporter function. *p*-Coumaric acid inhibited the uptake of nateglinide in a competitive manner. The K_i value of *p*-coumaric acid was estimated to be 2.1 mM. The estimated K_i value of *p*-coumaric acid was similar to that of ferulic acid (1.2 mM) (*24*).

It is interesting that caffeic acid has an inhibition manner on the uptake of nateglinide that is different from ferulic acid and *p*-coumaric acid despite the fact that all of them have similar structures. The different point between these substances is the functional group that exists in the meta position. It has not yet been reported which hydroxyl group affects the transport mechanism. However, it is suggested that the function of hydroxyl, which exists in the meta position, might affect the transport property of polyphenols or other drugs which are the substrates of nateglinide/fluorescein transporter in this study.

We then investigated the inhibitory effects of polyphenols on the uptake of fluorescein. Caffeic acid and *p*-coumaric acid inhibited the uptake of fluorescein. Although ferulic acid has been shown to inhibit competitively the uptake of nateglinide (*24*), ferulic acid stimulated the uptake of fluorescein. It has been reported that ferulic acid transport is much faster than that of fluorescein (*8*, *24*). It is possible that fast transport of ferulic acid caused the countertransport of fluorescein.

Absorption of drugs from the gastrointestinal tract is one of the important determinants for oral bioavailability. The recent development of molecular biological techniques has led to the identification of drug transporters responsible for the intestinal absorption of a wide variety of drugs (*34*). It is now widely recognized that drug transporters contribute to the absorption of administered drugs from the intestine. Since patients usually take many kinds of drugs at the same time in clinical, several drug interactions are caused due to the inhibition of transporters in the intestine. Drug−drug interactions involving peptide transporter 1 (PEPT1) and monocarboxylate transporters (MCTs) are likely to occur due to their broad substrate specificities. Besides PEPT1 and MCTs, we have to be aware of drug−drug interactions involving the fluorescein/H^+ cotransport system. Moreover, food−drug interactions may also occur at the transporter level in patients who have chronic diseases. This interaction is not known so much because people are not aware of the its dangerous possibility. It is important to be aware of the potential of food−drug interactions and to act in order to prevent undesirable and harmful clinical consequences.

It is possible that food−drug interactions involving the nateglinide/fluorescein transporter reduce the oral bioavailavility of drugs. The total intake of polyphenol is about 1 g/day. This amount is greater than the recommended daily dose of many kinds of drugs (10−100 mg/day) (*35*). To evaluate the inhibitory effect of total polyphenol on the nateglinide/fluorescein transporter system, it is important to elucidate every polyphenol on the nateglinide/fluorescein transporter. In this study, we focused on the differences of the substrate specificity of nateglinide/fluorescein transporter. Further studies are needed to elucidate the substrate specificity of the nateglinide/fluorescein transporter.

In summary, the results of this study suggest that the function of hydroxyl, which exists in the meta position, might affect the transport property of phenolic acids. The findings obtained in this study are useful to identify the substrate specificity of the fluorescein/H^+ transporter. Moreover, these results will be good knowledge for producing prodrugs and for interaction in the absorption level.

ABBREVIATIONS USED

PEPT1, peptide transporter 1; MCT, monocarboxylate transporter.

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