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Intestinal uptake of nateglinide by an intestinal fluorescein transporter

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Abstract

Nateglinide, a novel oral hypoglycemic agent, rapidly reaches its maximum serum concentration after oral administration, suggesting that it is rapidly absorbed in the intestine. However, nateglinide itself is not transported by MCT1 or PEPT1. The aim of this study was to characterize the transporters on the apical side of the small intestine that are responsible for the rapid absorption of nateglinide. It has been reported that the uptake of fluorescein by Caco-2 cells occurs via an H^+-driven transporter and that the intestinal fluorescein transporter is probably not MCT1. We examined the contribution of the fluorescein transporter to the uptake of nateglinide by Caco-2 cells. Fluorescein competitively inhibited H^+-dependent nateglinide uptake. All of fluorescein transporter inhibitors examined reduced the uptake of nateglinide. Furthermore, nateglinide inhibited fluorescein uptake. We conclude that the intestinal nateglinide/H^+ cotransport system is identical to the intestinal fluorescein/H^+ cotransport system.

Keywords: Nateglinide; Intestine; Monocarboxylate transporter; Fluorescein; Absorption; Caco-2

1. Introduction

Impairment of glucose-induced insulin secretion and insulin resistance are hallmarks of type 2 diabetes (non-insulin-dependent diabetes mellitus). To compensate for defective insulin secretion, sulfonylureas have been widely used for more than 40 years in the treatment of type 2 diabetes. However, sulfonylurea therapy has several disadvantages, such as excess hypoglycemia between meals, due to the long duration of action of these agents.

Nateglinide, a novel oral hypoglycemic agent, is a non-sulfonylurea anti-diabetic 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 [1–4]. The unique feature of nateglinide is rapid intestinal absorption and renal elimination. Because nateglinide is an anionic compound with p*K*a 3.1, it exists predominantly in ionized form at the intestinal physiological pH of 6.5. Moreover, its chloroform/water partition coefficient is reported to be 0.2 at pH 6.8, indicating that it is scarcely lipophilic. 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. However, nateglinide is not transported by monocarboxylate transporter 1 (MCT1/*SLC16A1*) [5]. Although nateglinide has a peptide-type bond in its structure, it has been reported not to be transported by peptide transporters (PEPT1/*SLC15A1*, PEPT2/*SLC15A2*) [6]. The transport mechanism of nateglinide in intestinal absorption has not been elucidated yet.

Caco-2 cells have been widely used to evaluate the oral absorability of drugs and to study their absorption mechanisms [7]. It has been reported that the uptake of fluorescein by Caco-2 cells occurs via an H^+-driven transporter and that the intestinal fluorescein transporter is probably not MCT1 [8]. The present study was carried out to characterize the transporters on the apical side of the small intestine that are responsible for the rapid absorption of nateglinide using

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Caco-2 cells. We examined the contribution of the fluorescein transporter to the uptake of nateglinide.

2. Materials and methods

2.1. Chemicals

Nateglinide and pravastatin were kindly donated by Yamanouchi (Tokyo, Japan) and Sankyo (Tokyo, Japan), respectively. Salicylic acid and benzoic acid were purchased from Wako Pure Chemical (Osaka, Japan). Fluorescein and L-lactic acid were purchased from Nacalai Tesque (Kyoto, Japan). All other reagents were of the highest grade available and used without further purification.

2.2. 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 [9]. For the uptake study, Caco-2 cells were seeded at a cell density of $1–3\times10^5$ cells/cm^2 on 12-well plastic plates (Corning Costar, Cambridge, MA). The cell monolayers were fed a fresh growth medium every 2 days and were used at 4 to 6 days for the uptake experiments.

2.3. Uptake studies in Caco-2 cell monolayers

The uptake experiment was performed as described previously [10]. Nateglinide uptake and fluorescein uptake were measured using monolayer cultures grown in 12-well plastic plates. The composition of the incubation medium was as follows: 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.4 mM KH_2PO_4, 0.3 mM NaH_2PO_4, 4.2 mM $NaHCO_3$, 25 mM D-glucose and 10 mM MES (pH 5.5, 6.0, 6.5 and 7.0) or 10 mM HEPES (pH 7.5 and 8.0). After the removal of the growth medium, cells were preincubated at 37 °C or 4 °C for 10 min with 1 ml of incubation medium. After the removal of the medium, 1 ml of incubation medium containing substrate was added. The monolayers were incubated for a stated time at 37 °C or 4 °C. Each cell monolayer was washed rapidly twice with an ice-cold incubation medium at the end of the incubation period. The cells were suspended in 0.6 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 12,000×g for 10 min.

2.4. Analytical procedures

Nateglinide was determined using an HPLC system equipped with a JASCO 880-PU pump 870-UV UV/VIS detector described previously [11]. The column was a Hitachi ODS Gel #3053 (4 mm i.d.×250 mm). A mobile phase containing 50 mM H_3PO_4 (pH 2.5):acetonitrile (55:45, v/v) was used. Column temperature and flow rate were 55 °C and 0.7 ml/min, respectively. The wavelength for the detection of nateglinide was 210 nm. The measurement of fluorescein was carried out in a spectrofluorometer (Hitachi 650-60) with an excitation wavelength of 490 nm and emission wavelength of 525 nm. Protein was measured by the method of Lowry et al. [12] with bovine serum albumin as a standard. Transporter-mediated uptake was determined as the difference between the uptake at 37 °C and that at 4 °C. Student's t-test was used for statistical analysis, and a value of $P<0.05$ 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).

3. Results

3.1. Characteristics of nateglinide uptake into Caco-2 cells

We investigated the characteristics of nateglinide uptake into Caco-2 cells. Fig. 1A shows the time course of the uptake of nateglinide. The uptake of nateglinide increased linearly over a period of 5 min. Fig. 1B shows the effect of extracellular pH on the uptake of nateglinide by Caco-2 cells. The uptake markedly increased with a decrease in extracellular pH. Fig. 1C shows the concentration-dependent uptake of nateglinide within the concentration range up to the solubility limit. The inset shows the Eadie-Hofstee plot. The Km and Vmax values were determined by kinetic analysis to be 0.24 mM and 34.6 nmol/mg protein/5 min, respectively.

3.2. Inhibitory effects of monocarboxylates on the uptake of nateglinide

Kuwayama et al. [8] reported that the uptake of fluorescein by Caco-2 cells occurs via an H^+-dependent carrier that has a wide spectrum of substrates and seems likely to be different from MCT1. The effects of fluorescein and various monocarboxylates on the uptake of nateglinide into Caco-2 cells in the presence of an inwardly directed H^+ gradient were examined. As shown in Table 1, fluorescein significantly reduced the uptake of nateglinide. Benzoic acid, pravastatin and salicylic acid reduced the uptake of nateglinide. On the other hand, L-lactic acid did not affect the uptake of nateglinide.

3.3. Dixon plot analysis of nateglinide uptake in the presence of fluorescein

The results of Dixon plot analysis of nateglinide uptake in the presence of fluorescein are shown in Fig. 2. Fluorescein was found to inhibit the uptake of nateglinide

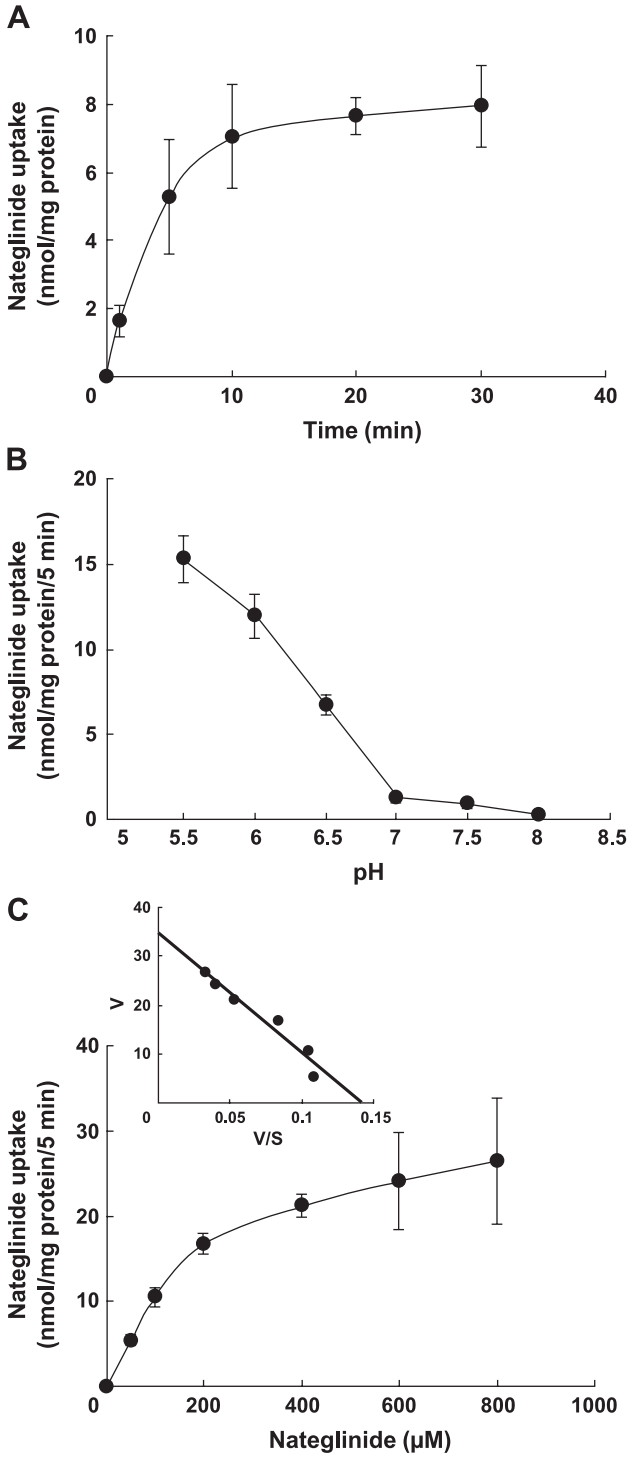

Fig. 1. (A) Time course of the uptake of nateglinide by Caco-2 cells. Cells were incubated for indicated periods with nateglinide (50 μM). (B) Effect of extracellular pH on the uptake of by Caco-2 cells. Cells were incubated in a medium at each pH containing nateglinide (50 μM). (C) Concentration dependence of the uptake of nateglinide by Caco-2 cells. Caco-2 cells were incubated for 5 min with various concentrations of nateglinide. Each point represents the mean with S.D. of 3–6 preparations.

Table 1
Effects of various compounds on the initial uptake of nateglinide by Caco-2 cells

Compound	Concentration (mM)	Nateglinide uptake (% control)
Control		100
Fluorescein	1	56.6±7.01**
Benzoic acid	10	48.2±1.72**
Pravastatin	10	76.0±2.68**
Salicylic acid	10	37.8±3.99**
L-Lactic acid	10	96.6±7.57

The uptake of nateglinide (50 μM) by Caco-2 cells was determined in the presence or absence of inhibitors. Each value represents the mean with S.D. of 3–6 preparations. The control value for the uptake of nateglinide was 12.3±2.12 nmol/mg protein/5 min.

** $P<0.01$, significantly different from the control.

competitively. The apparent Ki value calculated from Dixon plots for fluorescein was 0.65 mM.

3.4. Effect of nateglinide on the uptake of fluorescein by Caco-2 cells

In order to clarify whether the nateglinide/H^+ cotransport system is identical to that of fluorescein/H^+, the inhibitory effect of nateglinide on the uptake of fluorescein was investigated. As shown in Fig. 3, nateglinide significantly reduced the uptake of fluorescein by Caco-2 cells.

4. Discussion

Since nateglinide is absorbed rapidly from the intestine, it is likely to be absorbed via a specific transporter(s) [1–4].

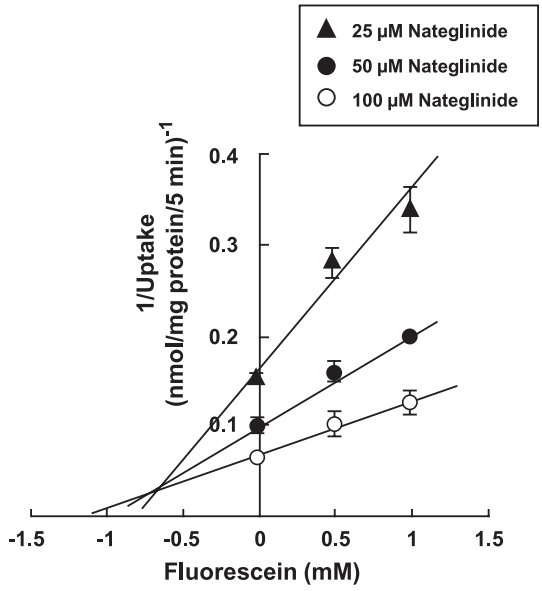

Fig. 2. Dixon plot of nateglinide uptake into Caco-2 cells in the presence of fluorescein. Uptake of 25 μM, 50 μM and 100 μM nateglinide was measured for 5 min with fluorescein. Each point represents the mean with S.D. of 3 preparations. The apparent Ki value was determined to be 0.65 mM by linear regression analysis from the Dixon plot.

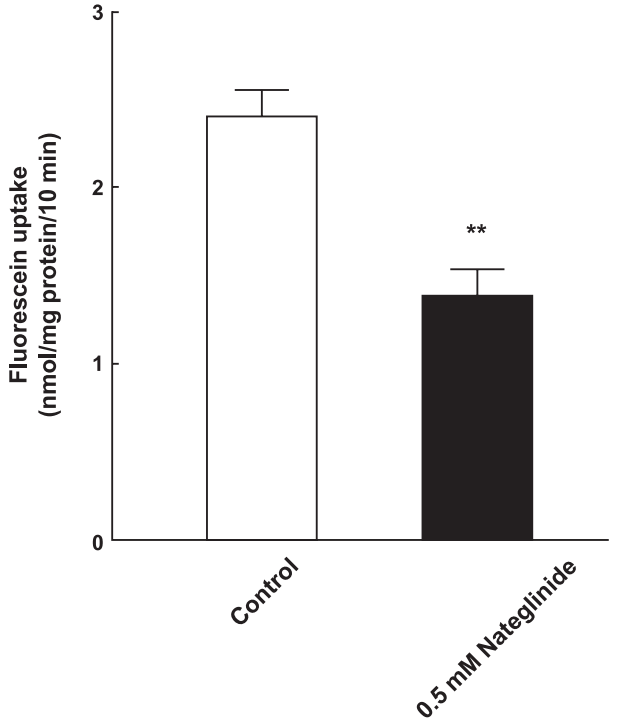

Fig. 3. Inhibitory effect of nateglinide on the uptake of fluorescein by Caco-2 cells. The uptake of fluorescein (50 μM) by Caco-2 cells was determined in the presence or absence of nateglinide. Each column represents the mean with S.D. of 6 preparations. $^{**}P<0.01$, significantly different from the control.

It has been reported that nateglinide is transported in an absorptive direction across Caco-2 cell monolayers, although nateglinide itself is not transported by PEPT1, PEPT2 or MCT1 [5,6]. The transport mechanism of nateglinide in intestinal absorption has not been elucidated yet. The aim of the present study was to try to determine transporters responsible for nateglinide transport.

Kuwayama et al. [8] reported that the uptake of fluorescein by Caco-2 cells occurs via an H^+-driven transporter. The transporter can transport fluorescein and salicylate, both of which have bulky aromatic rings, but not a small monocarboxylate. It is likely that the intestinal fluorescein transporter is not MCT1 and that MCT1-MCT4 are not involved in fluorescein transport. Since nateglinide also has bulky aromatic rings, it is possible that nateglinide is transported by an intestinal fluorescein transporter. In this study, we investigated whether an intestinal fluorescein transporter contributes to the uptake of nateglinide by Caco-2 cells.

We investigated the inhibitory effect of fluorescein on the uptake of nateglinide. Fluorescein was found to inhibit the uptake of nateglinide competitively, indicating that the efficient absorption of nateglinide is due to its fluorescein transporter-mediated absorption. We then examined the inhibitory effects of monocarboxylates on the intestinal absorption of nateglinide. It has been reported that salicylic acid, benzoic acid and pravastatin inhibit the uptake of fluorescein by Caco-2 cells [8]. All of them reduced the uptake of nateglinide. On the other hand, L-lactic acid, which is not an inhibitor of fluorescein transporter, did not affect the uptake of nateglinide. These results indicate that the substrate specificity of the nateglinide transporter is similar to that of the fluorescein transporter. In order to clarify whether the nateglinide/H^+ cotransport system is identical to the fluorescein/H^+ cotransport system, the opposite inhibitory effect of nateglinide on the uptake of fluorescein was investigated. Nateglinide significantly inhibited the uptake of fluorescein. Taking all of the results presented in this paper into consideration, we conclude that nateglinide shares a transporter with fluorescein. It is possible that fluorescein transporter-mediated nateglinide absorption is responsible for the rapid absorption of nateglinide.

Various MCT isoforms, each having a different tissue distribution, have been identified [13,14]. It has recently been demonstrated that at least five isoforms of MCT (MCT1, MCT2, MCT4, MCT5, and MCT8) are present in the small intestine and colon, but only MCT1–MCT4 have been characterized in terms of their substrates and inhibitor kinetics [13,15]. It has been reported that the uptake of nateglinide from the apical side is H^+-dependent and Na^+-independent [6]. H^+-dependent transport of monocarboxylate has been directly demonstrated for MCT1–MCT4. These MCTs exhibit affinities for those substrates and inhibitors with Km and Ki values of several mM. However, the estimated Km value for the saturable uptake of nateglinide was 0.24 mM. These findings suggest that MCT1-4 do not play a major role in the intestinal absorption of nateglinide. Moreover, the estimated Ki value of fluorescein for nateglinide uptake (0.65 mM) and the estimated Km value for the saturable uptake of nateglinide (0.24 mM) are similar to the Km value for the saturable uptake of fluorescein (0.4 mM) [8]. Our conclusion was further supported by these findings.

Dietary polyphenols have been widely assumed to be beneficial to human health by exerting various biological effects such as free radical scavenging, metal chelation, modulation of enzymatic activity and alteration of signal transduction pathways [16–18]. Epidemiological studies have shown relationships between consumption of polyphenol-rich foods and prevention of diseases such as cancer, coronary heart disease and osteoporosis, findings that have promoted interest in polyphenols [19–21]. There are a number of monocarboxylic acids among dietary polyphenols, especially in phenolic acids. They are commercially prepared and used as functional foods. It has been reported that several phenolic acids are transported by the intestinal fluorescein transporter [22–25]. It is well known that 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 drug–food interactions involving transporters. It is possible that a daily

diet of functional foods reduces the oral bioavailability of nateglinide. Results of functional analysis of fluorescein transporter should lead to the establishment of means for preventing drug–food interactions.

In summary, we have demonstrated that nateglinide shares a transporter with fluorescein. It is possible that fluorescein transporter-mediated nateglinide absorption is responsible for the rapid absorption of nateglinide. However, this transport system has not yet been elucidated at the molecular level. Further studies are needed to elucidate the mechanism of rapid intestinal absorption of nateglinide.

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