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Kinetic characterization of glycosidase activity from disaccharide conjugate to monosaccharide conjugate in Caco-2 cells

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Abstract

Glycosidase activity influences the intestinal absorption of glycosides. Our previous study in rats suggested that disaccharide conjugates might be prototypes for pre-prodrugs aiming at the Na^+/glucose co-transporter-mediated transport of prodrugs (drug glucoside) as a novel absorption pathway. One of the crucial factors is the formation of a glucoside drug from the disaccharide conjugate. Since there is a large species difference in metabolism, it is necessary to examine the cells and/or enzymes derived from human tissue to confirm this concept. In this paper, we kinetically characterized the glycosidase activity of disaccharide conjugates in Caco-2 cells. Disaccharide conjugates of p-nitrophenol (p-NP) (p-NP β-cellobioside, p-NP β-lactoside and p-NP β-maltoside) were hydrolysed to p-NP β-glucoside. β-glucosidase or β-galactosidase (lactase/phloridzin hydrolase, LPH) and α-glucosidase (sucrase–isomaltase) had different pH-dependent activities for disaccharide conjugates. At neutral pH, LPH has low affinity and low capacity, and sucrase–isomaltase has high affinity and high capacity, whereas at acid pH, LPH has high affinity and low capacity, and sucrase–isomaltase has low affinity and high capacity. The hydrolysis clearance calculated with Vmax/Km indicated that sucrase–isomaltase activity is much higher than LPH activity at either neutral or acid pH in Caco-2 cells. Since the hydrolysis rate of the disaccharide conjugate was highly dependent on the pH value and type of glycoside linkage, the appropriate selection of a glycoside form after consideration of these differences is the key to designing a sugar-conjugate prodrug.

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Introduction

The intestinal absorption of glycosides has been reported previously. Some glycosides are hydrolysed to aglycone, which is then absorbed, whereas others are absorbed as intact glycosides. Thus, glycosidase activity influences the intestinal absorption of glycosides, and this has recently been studied extensively (Hollman et al 1995; Andlauer et al 2000; Williamson et al 2000; Day et al 2003).

We also studied the intestinal absorption of disaccharide conjugates in rats, and reported that disaccharide conjugates of p-nitrophenol (p-NP), such as p-NP β-cellobioside, p-NP β-lactoside and p-NP β-maltoside, are hydrolysed to p-NP β-glucoside by glycosidases, and p-NP β-glucoside is actively transported by the Na^+/glucose co-transporter (SGLT1) in the rat small intestine (Mizuma 1998, 2002; Mizuma & Awazu 1998). The absorption clearance of p-NP β-glucoside, formed from p-NP β-maltoside by α-glycosidase catalysis, was similar to that of p-NP β-glucoside itself. In contrast, the absorption clearance of p-NP β-glucoside formed from p-NP β-cellobioside or p-NP β-lactoside by β-glycosidase (lactase/phloridzin hydrolase (LPH)) catalysis was higher than that of p-NP β-glucoside itself. We therefore hypothesized that cooperative vectorial transport by LPH and SGLT1 occurred.

These results are interesting from the viewpoint of not only basic science but also applied science because they suggest that β-disaccharides, such as p-NP β-cellobioside and p-NP β-lactoside, may be prototypes for pre-prodrugs aiming at the SGLT1-mediated transport of prodrugs (β-glucoside). Thereby, sugar conjugates may be useful for oral delivery, organ targeting or improved solubility. However, in general there is a large species difference in metabolism, that is a large difference in metabolic function

between humans and animals. Kinetic studies on these brush-border glycosidases from this particular human-derived cell type have not been reported in the literature, and it is therefore necessary to clarify glycosidase activity in cells derived from human tissue.

Caco-2 cells have been used to study the membrane permeability of drugs in the field of pharmaceutical science (Hidalgo et al 1989; Artursson & Karlsson 1991). However, the study of drug metabolic enzymes in Caco-2 cells is largely unreported because Caco-2 cells have been regarded as a membrane barrier model.

In this paper we studied glycosidase activity in the disaccharide conjugate to monosaccharide conjugate in Caco-2 cells, and kinetically characterized the glycosidase activity of these conjugates to clarify the applicability of Caco-2 cells to the study of sugar-conjugate absorption in humans.

Materials and Methods

Chemicals

p-NP β-glucoside, p-NP β-cellobioside, p-NP β-lactoside and p-NP β-maltoside were purchased from Sigma (St Louis, MO). Methanol and other chemicals were of reagent grade.

Hydrolysis of disaccharide conjugates (p-NP β-cellobioside, p-NP β-lactoside and p-NP β-maltoside) by Caco-2 cells

Caco-2 cells obtained from the American Type Culture Collection were cultured on a dish (9 cm^2) for 10 to 15 days according to the previous method (Mizuma et al 2002). Twenty minutes after placing the dish in an incubator at 37°C, the Caco-2 cell monolayer was washed with buffer five times, and 1.5 mL of drug solution warmed to 37°C was added to the Caco-2 cell monolayer. The drug solution (150 μL) was sampled at intervals over 60 min, and then transferred to a microtube (0.6 mL volume) containing a mixture of 50 μL of 10% perchloric acid, 50 μL of 0.1% Triton X100 and 50 μL of 1 mM 2,4-dihydroxybenzoic acid (internal standard) solution. The mixtures were centrifuged using a desktop centrifuge at 10 000 rpm for 5 min and the resultant supernatants were used in the HPLC assay.

Assay of p-NP β-glucoside, p-NP β-cellobioside, p-NP β-lactoside and p-NP β-maltoside

p-NP β-glucoside, p-NP β-cellobioside, p-NP β-lactoside and p-NP β-maltoside were determined by HPLC according to a previously reported method (Mizuma & Awazu 1998). The HPLC system consisted of a pump, UV detector (302 nm, 655 A-21, Hitachi) and integrator. The flow rate of the mobile phase was set at 1.5 mL min^{-1}. For the assay of p-NP β-cellobioside or p-NP β-maltoside, an ODS column (TSK gel ODS 80TM, 4.6 mm i.d., 150 mm length, Tosoh, Japan) and mobile phase consisting of 15% methanol and 0.05% phosphoric acid were used. An ODS column (Shodex C18-5B, 4.6 mm i.d., 250 mm length, Showa Denko, Japan) and mobile phase consisting of 22% methanol and 0.05% phosphoric acid were used in the p-NP β-lactoside experiment.

Data analysis

Kinetic parameters (Km, Vmax) for the hydrolysis of p-NP β-cellobioside, p-NP β-lactoside and p-NP β-maltoside to p-NP β-glucoside were obtained by fitting the data to the Michaelis–Menten equation using a non-linear least-squares fitting program, MULTI (Yamaoka et al 1981). Statistical analysis of the data was performed using Student's t-test.

Results and Discussion

Hydrolysis of p-NP β-cellobioside, p-NP β-lactoside and p-NP β-maltoside to p-NP β-glucoside

The production of p-NP β-glucoside from p-NP β-cellobioside, p-NP β-lactoside or p-NP β-maltoside was observed by Caco-2 cells cultured on a dish. Figures 1A–C show the time courses of p-NP β-glucoside formation from p-NP β-cellobioside, p-NP β-lactoside or p-NP β-maltoside, respectively, on the apical side of Caco-2 cells. The amount of p-NP β-glucoside increased with time. The formation rate of p-NP β-glucoside from p-NP β-maltoside was highest among these disaccharide conjugates, although the formation rates of p-NP β-glucoside depended on the concentrations of disaccharide conjugates (p-NP β-cellobioside, p-NP β-lactoside and p-NP β-maltoside). The hydrolysis rates of p-NP β-cellobioside were similar to those of p-NP β-lactoside at 1 and 5 mM.

Since p-NP β-glucoside is formed by the hydrolysis of the β-glucoside linkage between glucose and glucose in the cellobiose moiety of p-NP β-cellobioside, it is considered to be mediated by β-glucosidase, phloridzin hydrolase (LPH) (Lau 1987; Mantei et al 1988). Since p-NP β-glucoside is formed by the hydrolysis of the β-galactoside linkage between galactose and glucose in the lactose moiety of p-NP β-lactoside, it is considered to be mediated by β-galactosidase, lactase (LPH) (Lau 1987; Mantei et al 1988). In the same way, p-NP β-glucoside is formed by the hydrolysis of the α-glucoside linkage between glucose and glucose in the maltose moiety of p-NP β-maltoside, and it is considered to be mediated by α-glucosidase, maltase (Sorensen et al 1982) and/or sucrase–isomaltase (Gray et al 1979).

Hauri et al (1985) reported the expression of sucrase–isomaltase and LPH in Caco-2 cells, but no expression of maltase. Ogawa et al (2000) also reported the presence of the sucrase–isomaltase complex working as disaccharidase by Northern and Western analyses. Therefore, together with this report, these results indicate that p-NP β-cellobioside and p-NP β-lactoside were hydrolysed to p-NP β-glucoside by LPH in Caco-2 cells, and p-NP β-maltoside was hydrolysed to p-NP β-glucoside by sucrase–isomaltase in Caco-2 cells.

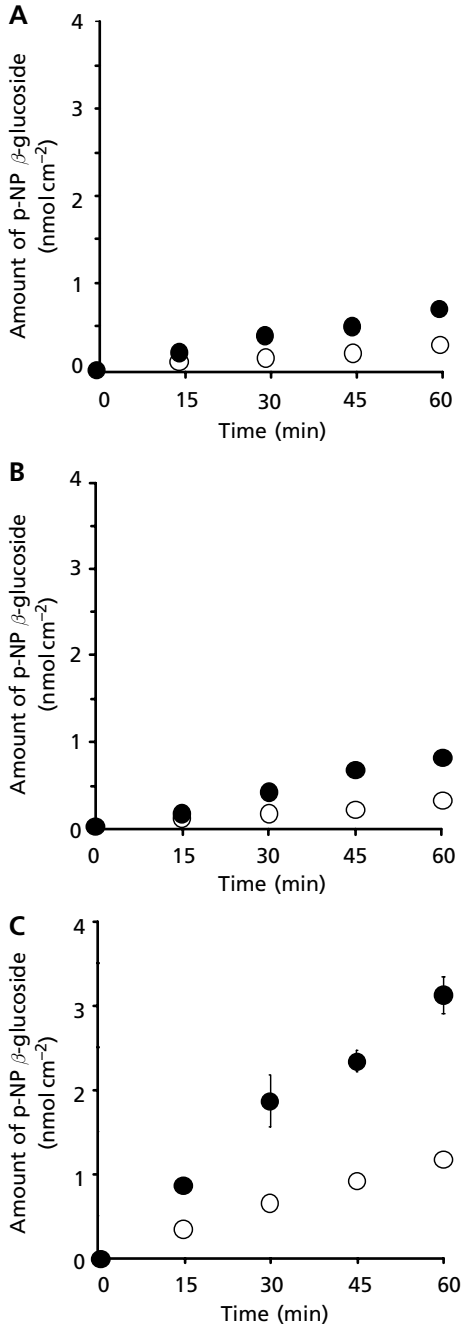

Figure 1 Time courses of p-NP β-glucoside production from p-NP β-cellobioside (A), p-NP β-lactoside (B) and p-NP β-maltoside (C). Concentrations of p-NP β-cellobioside, p-NP β-lactoside or p-NP β-maltoside: ○, 1 mM; ●, 5 mM. pH 7.4. Data represent the mean ± s.e.m. (n = 3).

Table 1 Comparison of kinetic parameters for the hydrolysis of disaccharide conjugate to monosaccharide conjugate by Caco-2 cells

Disaccharide conjugate	Apical pH	Vmax (nmol min^{-1} cm^{-2})	Km (mM)	Vmax/Km (μL min^{-1} cm^{-2})
p-NP	5.0	0.018 ± 0.001*	0.542 ± 0.189*	33.2×10^{-3}
β-cellobioside	7.4	0.048 ± 0.017	18.13 ± 9.44	2.65×10^{-3}
p-NP	5.0	0.061 ± 0.004*	1.67 ± 0.34*	36.5×10^{-3}
β-lactoside	7.4	0.078 ± 0.029	21.62 ± 11.25	3.61×10^{-3}
p-NP	5.0	1.78 ± 0.17*	2.82 ± 0.78	631×10^{-3}
β-maltoside	7.4	0.181 ± 0.006	5.12 ± 0.39	35.4×10^{-3}

Data represent the mean ± s.d. (n = 4). *$P < 0.005$ (vs pH 7.4).

Concentration and pH dependency of glycosidase activity for p-NP β-cellobioside, p-NP β-lactoside and p-NP β-maltoside

The formation rates of p-NP β-glucoside from p-NP β-cellobioside, p-NP β-lactoside and p-NP β-maltoside (0.25 to 10 mM) were concentration-dependent at pH 5.0 or 7.4, indicating Michaelis–Menten kinetics. The kinetic parameters for glycosidase activity for p-NP β-cellobioside, p-NP β-lactoside and p-NP β-maltoside at pH 5.0 and 7.4 are tabulated in Table 1. The order of the Vmax values was p-NP β-maltoside > p-NP β-lactoside > p-NP β-cellobioside at pH 7.4. Vmax values for p-NP β-cellobioside and p-NP β-lactoside were higher (2.7 times and 1.3 times, respectively) at pH 7.4 than at pH 5.0, whereas the Vmax value for p-NP β-maltoside at pH 7.4 was only one tenth of that at pH 5.0.

The order of the Km values was p-NP β-lactoside > p-NP β-cellobioside > p-NP β-maltoside at pH 7.4. All the Km values for p-NP β-cellobioside, p-NP β-lactoside and p-NP β-maltoside were higher (33 times, 13 times and 1.8 times, respectively) at pH 7.4 than at pH 5.0. These results indicate that at neutral pH, LPH has low affinity and low capacity, and sucrase–isomaltase has high affinity and high capacity, and at acid pH, LPH has high affinity and low capacity, and sucrase–isomaltase has low affinity and high capacity when LPH activity is compared with sucrase–isomaltase activity.

Moreover, all Vmax/Km values (equivalent to the clearance value) for p-NP β-cellobioside, p-NP β-lactoside and p-NP β-maltoside were more than 10 times higher at pH 5.0 than at pH 7.4. The Vmax/Km value for p-NP β-maltoside was highest among disaccharide conjugates at either pH value. The order of hydrolysis clearance calculated with the Vmax/Km was p-NP β-maltoside >> p-NP β-lactoside ≥ p-NP β-cellobioside at either pH value. In the rat small intestine clearances for glycosidase activities for p-NP β-cellobioside, p-NP β-lactoside and p-NP β-maltoside were 21.36 ± 0.25, 7.70 ± 0.03 and 102.96 ± 4.18 μL min^{-1} cm^{-1} (value ± s.d., n = 3–6), respectively, which were calculated from the data in the previous report (Mizuma & Awazu 1998). This indicates that the order of the hydrolysis activity is p-NP β-maltoside > > p-NP β-cellobioside > p-NP β-lactoside in the rat small intestine, which is similar to that in Caco-2 cells.

Based on these results, sucrase–isomaltase (α-glucosidase) activity is much higher than LPH (β-glucosidase or β-galactosidase) activity in Caco-2 cells. The glycosidase activity for disaccharide conjugate was highly dependent on the pH value and type of glycoside linkage, indicating

that the appropriate selection of glycoside form by considering these differences should be the key to designing a sugar conjugate prodrug.

In summary, Caco-2 cells have LPH and sucrase–isomaltase, hydrolysing disaccharide conjugates to glucose conjugates in Caco-2 cells, and the activities are dependent on the pH value and type of glycoside linkage. Caco-2 cells having these glycosidase activities should be useful for the study of the absorption of glycosides as natural products or prodrugs, and for confirming our hypothesis of cooperative vectorial transport of sugar conjugate by SGLT1 and LPH.

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