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Enhancement of Paracellular Transport of Heparin Disaccharide Across Caco-2 Cell Monolayers

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The enhancement of paracellular transport of heparin disaccharide using several absorption enhancers across Caco-2 cell monolayers was tested. The cytotoxicity of these enhancers was also examined. The enhancing effects by *Quillaja* saponin, dipotassium glycyrrhizinate, 18β-glycyrrhetinic acid, sodium caprate and taurine were determined by changes in transepithelial electrical resistance (TEER) and the amount of heparin disaccharide transported across Caco-2 cell monolayers. Among the absorption enhancers, 18β-glycyrrhetinic acid and taurine decreased TEER and increased the permeability of heparin disaccharide in a dose-dependent and time-dependent manner with little or negligible cytotoxicity. Our results indicate that these absorption enhancers can widen the tight junction, which is a dominant paracellular absorption route of hydrophilic compounds. It is highly possible that these absorption enhancers can be applied as pharmaceutical excipients to improve the transport of macromolecules and hydrophilic drugs having difficulty in permeability across the intestinal epithelium.

Key words: Heparin disaccharide, Caco-2 cell monolayers, Tight junction, Absorption enhancers, Paracellular transport

INTRODUCTION

Heparin, a naturally occurring acidic polysaccharide, is a potent inhibitor of blood coagulation, primarily through formation of a protease inhibitory complex with antithrombin III (Björk *et al.*, 1989). Although heparin is widely used as an anticoagulant in the clinic, heparin's other biological activities have been noted as well (Harenberg and Casu, 1996; Nelson *et al.*, 1993; Xie *et al.*, 2000). Furthermore, recent studies indicate that heparin/heparan sulfate disaccharides generated by heparinases, can downregulate the production of tumor necrosis factor (TNF)-α by macrophages *in vitro*, and arrest immune cell-mediated delayed-type hypersensitivity and arthritis reactions *in vivo* (Cahalon *et al.*, 1997). Furthermore, these disaccharides can modulate the adhesion and migration of human T cells to both extracellular matrix and immobilized fibronectin (Hershkoviz *et al.*, 2000). Heparin/heparan sulfate disaccharides can regulate a spontaneous secretion of IL-8 and IL-1β as well as TNF-α from intestinal epithelial cells (Chowers *et al.*, 2001). Such findings strongly suggest that heparin disaccharides may act as immunoregulators with therapeutic effects.

Oral administration of heparin disaccharide is essentially ineffective, because its hydrophilicity and high charge density preclude its efficient absorption from the gastrointestinal tract (unpublished data). The intestinal epithelium represents a major barrier to the absorption of orally administered drugs and nutrients into systemic circulation. The transport of molecules across the intestinal epithelium occurs by passive diffusion through transcellular or paracellular route, and through carrier-mediated active or facilitated transport. But the intestinal absorption of hydrophilic and high molecular weight drugs is profoundly limited by their physicochemical characteristics. Hence, the intestinal epithelium is a significant barrier for hydrophilic molecules because they cannot easily transverse the lipid bilayer of the cell membrane (Anderson and van Itallie, 1995). The paracellular pathway is a dominant path-

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way for the passive transepithelial solute flow of hydrophilic compounds in the small intestine, and its permeability depends on the regulation of the intercellular tight junctions. Accumulated findings indicate that the intercellular junctions of epithelia are dynamic structures that can modulate their permeability in response to various stimuli such as hormones and nutrients (Fasano, 1998). The reversible opening of the intercellular spaces is of considerable interest in pharmaceutics since it shows a way by which the absorption of more hydrophilic drugs can be increased.

To increase the paracellular transport of less absorbable drugs, the use of absorption enhancers has been studied. Numerous classes of compounds with diverse chemical properties have been reported to enhance the intestinal absorption of hydrophilic compounds, including detergents, surfactants, bile salts, Ca^{2+} chelating agents, fatty acids, modified polysaccharides, phospholipids *etc.* (Ward *et al.*, 2000). Recently, several reports have been published that heparin in combination with the sodium *N*-[8(-2-hydroxybenzoyl)amino]caprylate (SNAC) produced significant elevations of the intestinal absorption of heparin in rats (Leone-Bay *et al.*, 1998), primates (Rivera *et al.*, 1997) and human (Baughman *et al.*, 1998) as well as *in vitro* (Brayden *et al.*, 1997).

Although enhancers could increase the paracellular permeability of the hydrophilic compounds by disrupting the structure of the lipid bilayers or tight junctions, it seems that epithelial damage and cytotoxicity are unavoidably connected with the opening of the tight junctions. In this report, we determine the effects and cytotoxicity of absorption enhancers that can increase the paracellular transport of a heparin disaccharide (MW 665 as the sodium salt), the major repeating unit of heparin, across Caco-2 cell monolayers.

MATERIALS AND METHODS

Materials

Caco-2 cells were purchased from American Type Culture Collection (Rockville, MD, U.S.A.). Dulbeccos modified Eagle's medium (DMEM), Dulbeccos phosphate buffered saline (DPBS), non-essential amino acid (NEAA), trypsin and disodium ethyenediaminetetraacetate (EDTA), collagen type I, penicillin-streptomycin (10,000 units/ml and 10 mg/ml in 0.9% sodium chloride, respectively), Hanks balanced salt (HBSS), glucose, *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid] (HEPES), 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), *Quillaja* saponin, taurine and 18β-glycyrrhetinic acid were purchased from Sigma (St Louis, MO, U.S.A.) or Aldrich (Milwaukee, WI, U.S.A.). Dipotassium glycyrrhizinate was supplied by Alps Pharm. (Tokyo, Japan) and fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, U.S.A.). Other reagents and chemicals were of the best grade available.

High-performance liquid chromatography (HPLC) was performed on a 5 μm particle size strong-anion exchange (SAX) analytical column from Phenomenex (Torrells, CA, U.S.A.) of dimension 0.46 × 25 cm using ÄKTA purifier controlled by UNICORN software 3.1 from Amersham Pharmacia (Uppsala, Sweden).

Cell culture

Caco-2 cells between passage 31 and 49 were cultured routinely in DMEM, pH 7.4, supplemented with 1% non-essential amino acid, 10% FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin at 37°C in an atmosphere of 95% air, 5% CO_2 and 90% relative humidity. Cells were harvested by treatment with trypsin-EDTA and resuspended in DMEM before reaching the confluence. They were finally seeded at a density of about 2.5 to 3.0 × 10^5 cells/ml on collagen-coated Transwell® polycarbonate filters (surface area = 1 cm^2, pore size = 3.0 μm) from Costar (Cambridge, MA, U.S.A.). Media in the apical and basolateral chambers were changed every other day and cells were allowed to reach the confluence for 21 days before the experiment.

Preparation of heparin disaccharide

Heparin was depolymerized with heparinase I prepared from *Bacteroides stercoris* HJ15 as described previously (Kim *et al.*, 2000). The depolymerization mixture® was freezedried and size-fractionated on Bio Gel P-10 column (2.5 × 110 cm) equilibrated with 0.1 M NaCl. The main fraction of the disaccharide was desalted by Bio-Gel P-2 chromatography and freeze-dried. Its homogeneity was examined by HPLC and the structure was characterized by ^{1}H-NMR as described previously (Merchant *et al.*, 1985).

Measurement of transepithelial electrical resistance (TEER)

Cell monolayers were treated on the apical side with absorption enhancers of various concentrations dissolved in HBSS containing 11 mM D-glucose and 25 mM HEPES, pH 7.4 for 20 min and washed with the same media. The effects of absorption enhancers on TEER across Caco-2 monolayers were measured with a Milicell® electrical resistance system from Milipore Corp. (Bedford, MA, U.S.A.) before and after the application of enhancers. The resistance due to cell monolayers was determined and the results were presented as the percentage of the initial (t = 0) value in the same monolayers. A change of TEER was

examined for 4 h after washing absorption enhancers with HBSS. Control values were measured in the range of 500 -600 $\Omega \cdot cm^2$.

Assesment of paracellualr permeability of heparin disaccharide

Media in the apical side were replaced with fresh HBSS and preincubated for 1 h at 37°C. Transport studies were performed on Caco-2 cell monolayers with or without the enhancers at 37°C. Enhancers in HBSS (0.5 ml) were applied to the apical side of the monolayers for 20 min to evaluate the reversibility of their enhancing effects and adjust to the physiological incubation conditions. Samples were taken from the basolateral side every 1 h up to 4 h. The amount transported across Caco-2 cell monolayers was analyzed by SAX-HPLC as described above. The apparent permeability coefficients were calculated using the following equation: $P_{app} = (dQ/dt) \cdot (1/A) \cdot (1/C_0)$, where dQ/dt (mol transported/sec) is the permeability rate of heparin disaccharide across Caco-2 cell monolayers, A (cm^2) represents the diffusional surface area of the insert, and C_0 (M) denotes the initial concentration of heparin disaccharide in the apical side. All measurements were determined in triplicate and expressed as mean ± SD.

Evaluation of cytotoxicity of enhancers

Cytotoxicity of enhancers tested in this report was evaluated by MTT assay (Liu *et al.*, 1999) and the determination of lactate dehydrogenase (LDH) activity (Choksakulnimitr *et al.*, 1995). Mitochondrial dehydrogenase (MDH) activity was measured for MTT assay. The percentage of LDH released was calculated by: % $LDH_{release} = LDH_{medium}/(LDH_{medium} + LDH_{detached\ cell} + LDH_{cell}) \times 100$.

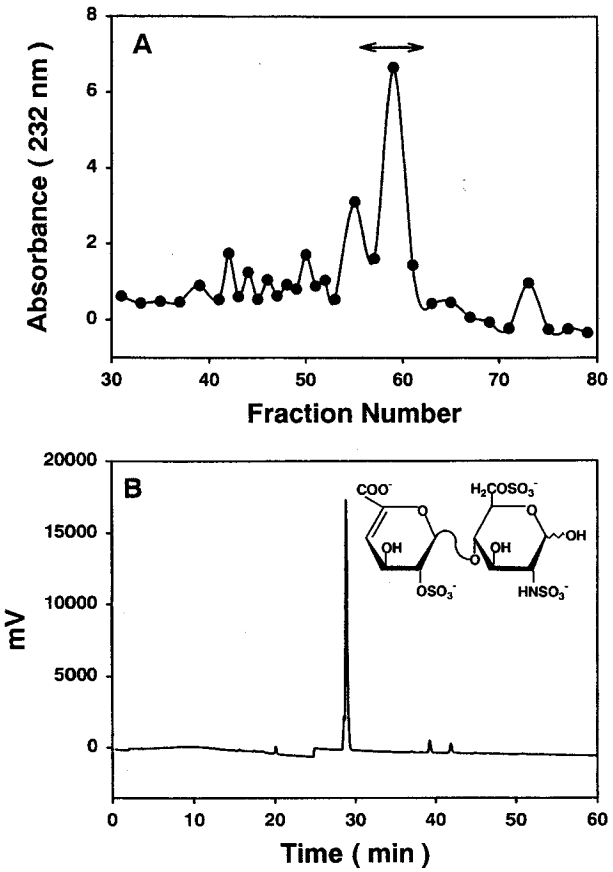

Fig. 1. Isolation and analysis of heparin disaccharide. Heparin (2 g) was depolymerized with 2 U heparinase I prepared from *Bacteroides stercoris* HJ15 in 50 mM phosphate buffer containing 50 mM KCl for 40 hrs. (A) The depolymerization mixture was freeze-dried and size-fractionated on a Bio-Gel P-10 column (2.5 × 110 cm) equilibrated with 0.1 M NaCl. The main fraction indicated by arrow was collected and desalted by Bio-Gel P-2 column. (B) Analytical SAX-HPLC analysis of heparin disaccharide. The inset indicates the structure of heparin disaccharide.

RESULTS

Preparation of heparin disaccharide

Heparin disaccharide from the heparin digestion mixture with heparinase I was isolated and characterized (Fig. 1). This heparin disaccharide represents a model compound for the hydrophilic substances transported through a paracellular route only.

Effects of absorption enhancers on TEER

The influence of absorption enhancers on TEER across Caco-2 cell monolayers was monitored as a function of time at several concentrations. Most of absorption enhancers showed a dose-dependent and time-dependent effect on TEER, an indicator for the opening of tight junctions (Fig. 2). *Quillaja* saponin decreased TEER drastically even at a very low concentration. The treatment with saponin showed almost the same value as the control at the concentration of 0.005%, while TEER showed a drastic decrease on treatment with more than 0.025% saponin. In the presence of 0.01% saponin, a transient decrease of up to 40% of the initial TEER was observed but the TEER recovered to more than 60% of the initial value within 1 h following the removal of enhancers. At a concentration above 0.05%, the saponin induced an immediate and sustained decrease in TEER, suggesting the complete disruption of cell monolayers. Such a result was comparable to the treatment of cell monolayers with 1% Triton X-100, the positive control. The TEER gradually decreased in a time-dependent and dose-dependent manner when taurocholate was used within the range of 0.02 to 0.1%. But at the concentrations of more than 0.2%, it induced an immediate and sustained decrease in TEER within 1 h (Fig. 2A).

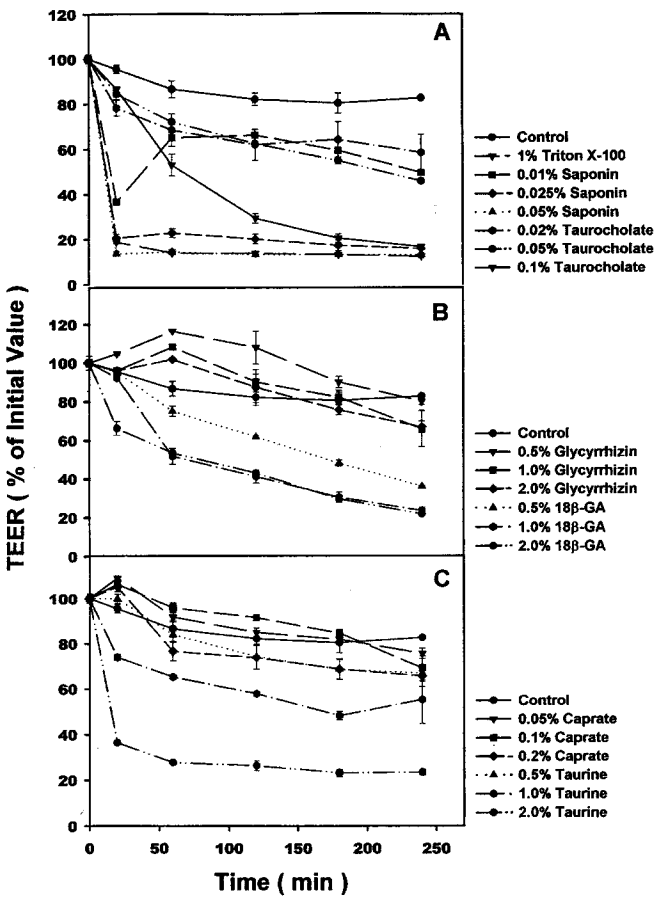

Fig. 2. Effect of absorption enhancers on TEER across Caco-2 cell monolayers. Enhancers were applied to the apical side of the monolayers at various concentrations for 20 min. The TEER was measured according to MATERIALS AND METHODS. Each value represents mean ± SD (n = 3).

On treatment with dipotassium glycyrrhizinate and its major metabolite, 18β-glycyrrhetinic acid, the change of TEER showed in different patterns. In contrast to other enhancers, dipotassium glycyrrhizinate increased TEER within 2 h, while 18β-glycyrrhetinic acid gradually dropped TEER in a dose-dependent and time-dependent manner (Fig. 2B). When the Caco-2 cells were treated with 0.05 and 0.1% caprate, the TEER was not significantly changed. But the treatment with 0.2% caprate resulted in a transient increase of TEER and then it was gradually decreased to 80% of the control level. In case of taurine, the TEER declined proportionally to the taurine concentration and the incubation with 2% taurine led to an immediate decrease of 60-70% after the 20 min. The effect persisted up to 4 h in the presence of 2% taurine on the apical side (Fig. 2C).

Effects of absorption enhancers on the transport of heparin disaccharide

After treatment with several absorption enhancers for 20 min, the transport of heparin disaccharide across Caco-2 cell monolayers was increased as shown in Table I. When the cell monolayers were treated with 1% Triton X-100 or 0.05% saponin, most of heparin disaccharide in the apical side was transported, indicating a complete disruption of cell monolayers. But at lower concentrations, saponin showed a time-dependent and dose-dependent effect and the addition of 0.01% saponin was similar to the control level showing no increase of permeability. The treatment of dipotassium glycyrrhizinate between 0.5 and 2.0% also showed an absorption enhancing activity and increased the apparent permeability coefficient P_{app} by 4.4- to 8.8-fold compared to the control. Interestingly, its major metabolic compound, 18β-glycyrrhetinic acid, also increased the P_{app} ratio to the control at the same concentration by 11.1-

Table I. Effect of absorption enhancers on the transport of heparin disaccharide

Enhancers (%)	Transport amount (%)	P_{app} (10^{-6} cm/sec)	P_{app} ratio to control
Control	0.32	0.23	1
1% Triton X-100	98.45	34.67	151
Saponin			
0.01	0.32	0.23	1
0.025	28.40	29.97	130
0.05	96.55	33.36	145
Taurocholate			
0.02	3.19	1.81	9.8
0.05	3.11	2.25	7.9
0.1	11.38	18.58	80.8
Dipotassium glycyrrhizinate			
0.5	0.50	1.00	4.4
1.0	0.74	1.81	7.9
2.0	1.29	2.02	8.8
18β-Glycyrrhetinic acid			
0.5	1.22	2.56	11.1
1.0	3.22	4.31	18.7
2.0	3.25	5.61	24.4
Caprate			
0.05	0.18	0.23	1
0.1	0.25	0.35	1.5
0.2	0.42	0.53	2.3
Taurine			
0.5	0.88	0.44	1.9
1.0	5.35	13.42	58.4
2.0	14.17	19.06	82.9

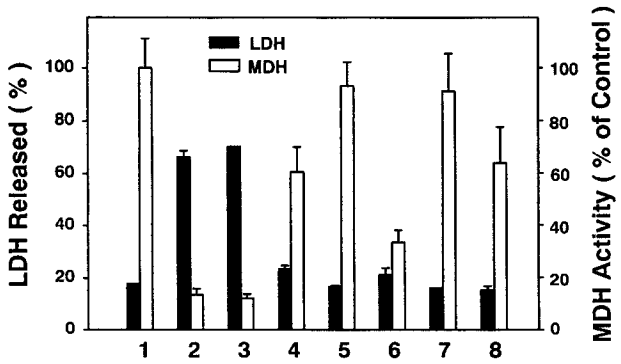

Fig. 3. Effect of enhancers on LDH released and MDH activity from Caco-2 cell monolayers. 1. control; 2. 1% Triton X-100; 3. 0.05% saponin; 4. 0.1% taurocholate; 5. 2% glycyrrhizin; 6. 18β-glycyrrhetinic acid; 7. 0.2% caprate; 8. 2% taurine. Each value represents mean ± SD (n=3).

to 24.4-fold. When caprate and taurine were used as enhancers, the P_{app} was also increased in a dose-dependent and time-dependent manner. However, the addition of caprate lower than 0.1% caused neither a significant increase of permeability of heparin disaccharide nor a significant decrease in TEER. At the concentration of 0.2 %, the P_{app} ratio to the control was only increased by 2.3-fold in contrast to previous reports (Sakai *et al.*, 1998). In the presence of 1.0 and 2.0% taurine, the transport amount of heparin disaccharide was increased to approximately 5.4 and 14.2% and the P_{app} ratio to the control was also increased by 58- and 83-fold, respectively.

Cytotoxicity of absorption enhancers

Most enhancers tested with the exception of *Quillaja* saponin only released low levels of LDH comparable to the control values. When cells were incubated with 0.05% saponin, the released LDH activity reached 70%, which is comparable to the value of 1% Triton X-100 treatment (Fig. 3). MDH activity obtained from MTT assay was also considerably reduced at the concentration of 0.05% saponin. Both LDH release and MTT assays indicate that enhancers such as dipotassium glycyrrhizinate, taurine and caprate have no effects on the plasma membrane and cell viability (Fig. 3).

DISCUSSION

The paracellular route is a dominant pathway for the passive transepithelial solute flow of hydrophilic compounds in the small intestine, and its permeability depends on the regulation of intercellular tight junctions (Anderson and van Itallie, 1995). The paracellular pathway is defined as the space between the cells and comprises both tight junctions and lateral intercellular spaces. The tight junction also contributes to epithelial transport by its involvement in epithelial surface polarity, maintaining the distinct apical and basolateral surface compositions necessary for vectorial transport across epithelia.

The tight junction constitutes the principal barrier to the passive movement of fluids, electrolytes, macromolecules and cells through the paracellular pathway and presents one of the major problems limiting the effective use of parentral route for the administration of macromolecules such as proteins, peptide and sugars (Clarke *et al.*, 2000). However, evidence suggests that the constituent cells of many epithelia are capable of regulating tight junction structure and permeability by factors of potential physiological and pathological importance including cyclic nucleotides (Duffey *et al.*, 1981), intercelluar calcium concentrations (Palant *et al.*, 1983), transient mucosal osmotic loads (Madara, 1983), G-protein (Hopkins *et al.*, 2000) and protein kinase C (Stenson *et al.*, 1993). The tight junction is subject to physiological and pathological regulation and undergoes dynamic modulation by agents as diverse as phorbol esters (Clarke *et al.*, 2000), cytokines (Walsh *et al.*, 2000), calcium (Palant *et al.*, 1983), cytochalasin D (Madara *et al.*, 1986) and bacterial toxins (Fasano and Uzzau, 1997).

This study demonstrated that several absorption enhancers could make heparin disaccharide permeate through Caco -2 cell monolayers. Because of its structural features, the transport of this molecule through the membrane is very restricted. Absorption enhancers with little cytotoxicity were required to improve the heparin disaccharide's low permeability across the intestinal membrane through widening the tight junctions. More selective and less toxic ways to regulate the paracellular permeability have to be found before this strategy can be applied in pharmaceutical formulations for heparin oral delivery.

Saponin from *Quillaja* bark was reported as a membrane-perturbing compound that can increase the cell membrane permeability (Pillion *et al.*, 1996). As expected, the saponin rapidly decreased TEER but its cytotoxicity was very severe. Below the concentration of 0.005%, saponin transiently decreased TEER but showed no significant absorption enhancing effect.

Caprate and dipotassium glycyrrhizinate showed no significant cytotoxicity through LDH and MTT assays. The permeability enhancing effects of these agents, however, were also low when compared to other enhancers. Although compounds, such as glycyrrhizinate salt and caprate, have been used extensively as permeation enhancers, our results suggest that they play no significant role in the intestinal permeability. According to a recent report, a single treatment of dipotassium glycyrrhizinate had little effect on the transepithelial transport of hydrophilic and hydrophobic model compounds and it did not reduce TEER of Caco-2 cell monolayers (Sakai *et al.*, 1998). Another

report indicated that the combined use of sodium deoxycholate and dipotassium glycyrrhizinate had a synergistic effect, conceivably by dipotassium glycyrrhizinate enhancing the activation of protein kinase C by sodium deoxycholate (Sakai *et al.*, 1999). We demonstrated that dipotassium glycyrrhizinate has a small modulating effect on the tight junctions. The 18β-glycyrrhetinic acid, a metabolite of dipotassium glycyrrhizinate, showed a larger absorption enhancing activity in the transport of hydrophilic compounds. It increased the permeation of heparin disaccharide by a 3-fold higher level than did the dipotassium glycyrrhizinate, with little observed cytoxicity.

Of all the enhancers used in this experiment, taurine showed the lowest cytotoxicity as well as the highest enhancing effect for the transport of heparin disaccharide. It improved transport by 14% and P_{app} up to 19-fold over control, at a 2% concentration. Taurine is a naturally occurring amino acid present at high concentrations in mammalian tissues including liver. One of its possible functions may involve the modulation of Ca^{2+} transport. In many tissues, taurine has been shown to have a marked effect on kinetics of Ca^{2+} movement across the membranes of both cellular and subcellular preparations. The action of taurine on both low and high levels of intracellular Ca^{2+} has been ascribed to direct modulation of Ca^{2+} channels or an indirect effect operating through Na^{+}/Ca^{2+} exchange (Palmi *et al.*, 1999). There are reports that Ca^{2+} plays a role in the regulation of the tight junction by activation of protein kinase C caused by capric and lauric acid (Lindmark *et al.* 1998; Ma *et al.*, 1999). We speculate that taurine exerts its absorption enhancing effect through the modulation of cellular Ca^{2+} transport.

The increase in the concentration of enhancers would inevitably provoke the cytotoxicity but such enhancers have been used as food or pharmaceutical additives at the almost same concentrations. The present study has revealed that these absorption enhancers might be useful to enhance the intestinal permeability for the hydrophilic compounds, such as sulfated sugars including heparin. A high charge density and large molecular size (average molecular weight 12,000-15,000) of heparin result in a very low absorption through the gastrointestinal tract (Hiebert *et al.*, 2000). When these absorption enhancers are used in practice, it is essential that they do not adversely affect the membrane integrity of the epithelium. Further research is required to demonstrate not only the determination of an appropriate concentration for the application of these enhancers, but also their mechanism of action and cytotoxicity.

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