Transcellular and Lipophilic Complex-Enhanced Intestinal Absorption of Human Growth Hormone

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Purpose. To evaluate the transcellular mechanism of novel enhancers absorption enhancement of human growth hormone (hGH), by examining the involvement of a P-glycoprotein-like efflux system, changes in membrane fluidity, and membrane damage.

Methods. Caco-2 cell monolayers were grown on Snapwell® filter supports and placed in a side-by-side diffusion apparatus. Transport in both the apical to basolateral (AP to BL) and basolateral to apical (BL to AP) direction was measured at different temperatures and in the presence of potential inhibitors. Fluorescence anisotropy measurement was used to measure membrane fluidity. The fluorescence anisotropy of DPH- and TMA-DPH-labeled cell suspensions was measured at room temperature. LDH (a measure of cytosolic lactate dehydrogenase) leakage assay was used to evaluate cytotoxicity.

Results. The bi-directional transepithelial fluxes of hGH in the presence of these novel enhancers across Caco-2 cells showed marked asymmetry. Average permeability coefficient values obtained in the apical to basolateral (AP to BL) direction were lower than those of the reverse (BL to AP) direction. On the other hand, the fluxes for hGH alone were symmetric. When P-gp-like efflux inhibitors were included in the transport medium, the permeability coefficient value of BL to AP direction was significantly decreased while the transport was increased in the reverse direction in the presence of novel enhancers. In addition, lowering the temperature to 25°C completely eliminated the asymmetry of hGH transport in the presence of novel enhancers. It was also shown by fluorescence anisotropy that these novel enhancers alone only slightly increased membrane fluidity. On the other hand, upon addition of hGH to the novel enhancers, the cell membrane showed a dramatic change as compared to treatment with novel enhancers alone. The results from the LDH assay showed that the novel enhancers and/or hGH did not cause cell damage, at least up to 1 hour, and the damage seen at the 2 hour point is also much lower than other known enhancers.

Conclusions. This study shows that human growth hormone alone cannot be transported across Caco-2 cells, except in small quantities, by passive diffusion, but in the presence of novel enhancers, human growth hormone permeation is substantial. In addition, the asymmetry of transport of the complexed hGH appears to be due to a P-gp-like efflux system.[3] Assuming that the present substrate specificity of the P-gp-like efflux system shows the same preference for hydrophobic molecules as p-gp, the present work also indirectly shows that human growth hormone has become more lipophilic in the presence of these novel enhancers. Furthermore, membrane fluidity data also supports the premise that these novel enhancers interact and stabilize hGH, to make them more hydrophobic and easier to be transported through cell membranes.

KEY WORDS: human growth hormone; Caco-2 cells; P-gp-like efflux system; membrane fluidity; novel enhancer.

INTRODUCTION

Many proteins and peptides are potent endogenous molecules and specific in producing their pharmacological effects. Unfortunately, development of these drugs has been hampered by the difficulty in delivering them to their target sites. To deliver these peptide and protein drugs by routes other than parenteral is problematic because of their physicochemical properties, e.g., size, charge and hydrophilic characteristics, as well as their tendency to undergo metabolism at epithelial barriers (1). Therefore, numerous classes of synthetic or natural absorption enhancers, have been used in many peptide and protein formulations.

Most oral absorption enhancement approaches are aimed at causing disruption of the cell membrane, e.g., surfactants, or intercellular junctions, e.g., ethylenediamine tetraacetic acid (EDTA), cytochalasins, and sodium caprate (2). These agents are generally non-selective and therefore, when used in large quantity, can cause cell death. Emisphere Technologies, Inc. has developed several series of novel enhancers that are derivatives of amino acids with low molecular weights (MW < 300) and demonstrated that they can effectively enhance absorption of drugs that are traditionally administered only by injection. It was earlier shown that these novel enhancers increased absorption of several macromolecules *in vivo* in rats and primates, such as salmon calcitonin (3), interferon-α (4), and human growth hormone (hGH) (5). It has been demonstrated that enhancement was not due to metabolizing enzyme inhibition, and was not through classical penetration enhancement, e.g., pretreatment of tissue with enhancers has no effect and the enhancers show specificity (6). Thus, while absorption of salmon calcitonin and interferon-α was increased by a specific novel enhancer, heparin absorption, with the same enhancer, was not (7). It was also found (6) that while the permeability of hGH increased with addition of some of these novel enhancers, there was no effect of these same enhancers on mannitol, a paracellular marker; progesterone, a transmembrane marker; and thyrotropin releasing hormone (TRH) in isolated rabbit intestinal tissue. Furthermore, it was shown that while the permeation rate of hGH was the same from mucosal to serosal or serosal to mucosal in isolated tissue specimen, the permeation rate of hGH in the presence of these novel enhancers for AP to BL direction is much lower than that in the BL to AP direction in a Caco-2 cell line.

P-glycoproteins (P-gps) are the best known effluxers present in several cancer cell lines, and it is well documented that P-gps are overexpressed in Caco-2 cells. The main characteristic among P-gp substrates is that the substrates are usually amphiphilic, so that these drugs can partition into and out of the membrane in a rapid equilibrium, but diffuse slowly, in a rate limiting step, through the bilayer of the non-polar fatty acid side chains (8). These drugs are then recognized by an energized multidrug transporter as a potential target when they are present within the lipid bilayer and are removed directly from the plasma membrane. Protein drugs are generally very hydrophilic

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[3] Because it is lack of definitive evidence that the efflux system presented in this paper is a P-glycoprotein, the authors will address the efflux system as a P-gp-like efflux system throughout.

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and such protein drugs are not substrates for P-gps. The only possibility for a protein being recognized by a P-gp or a P-gp-like efflux system is that it is not in its native conformation, but in a stable intermediate conformation which has been widely reported in the literature and are known to expose their hydrophobic side chains or patches. Therefore, we hypothesized that there are specific interactions between the effective novel enhancers and hGH causing some conformational changes in hGH, i.e., they become more hydrophobic.

This hypothesis also suggests that these complexes will passively diffuse through lipid bilayers of the cell membrane, and in turn, the fluidity of cell membranes will be changed. Several classes of absorption enhancers have been shown to increase the permeability to drugs through isolated intestinal membranes, cultured cell membranes, and synthetic lipid bilayers, by inducing a change in the membrane lipid structure (10,11). For example, salicylate was shown to perturb isolated epithelial cell membranes and increase release of the impermeable fluorescein dye and β-lactam antibiotics (12). Kajii *et al.* (13) also found that sodium salicylate and sodium caprylate interact with membrane proteins, cause membrane perturbation and enhance permeability. Diphenylhexatriene (DPH) and trimethyl-ammonium diphenylhexatriene (TMA-DPH) are commonly used to monitor membrane lipid status. The fluorescence anisotropy of these two probes reflects membrane fluidity of the cells. DPH is considered to label the interior of the lipid layer, while TMA-DPH labels the exterior of the lipid bilayer situated closer to the aqueous interface. Leakage of the cytosolic enzyme-lactate dehydrogenase (LDH) into the bathing medium is also a good indicator of membrane integrity.

The purpose of this study is to provide additional mechanistic insight as to how the novel enhancers increase tissue absorption of hGH. Specifically if the novel enhancer-induced absorption of hGH is dependent on P-gp-like efflux system, but hGH alone is not, it would support the concept that the complex is sufficiently lipophilic to be a substrate for P-gp-like efflux system. More direct evidence can be found by examining lipid bilayer fluidity.

MATERIALS AND METHODS

Materials

The Caco-2 cell line (passage 17) was obtained from American Tissue Culture Collection, Rockville, MD. Dulbecco's Modified Eagle's medium (DMEM) containing 4.5 g/L glucose and 1% L-glutamine, heat-inactivated fetal bovine serum (FBS), non-essential amino acids (NEAA, ×100), and a penicillin and streptomycin mixture was obtained from Gibco, Grand Island, NY. Snapwell® plates with inserts (12 mm diameter and 0.4 μm pore size) were obtained from Fisher Scientific, St. Louis, MO. The side-by-side diffusion chambers, gas manifold and block heater were from Precision Instrument Design, Los Altos, CA. [^{125}I]-human Growth Hormone (100–102 μCi/μg) was purchased from NEN DuPont Research Products, Boston, MA. [^{3}H]-Vinblastine (15.5 Ci/mmol) was from Amersham Life Science, Arlington Heights, IL, and [^{14}C]-Mannitol was obtained from Sigma Chemical, St. Louis, MO. Novel enhancers (E352 and E414) (Fig. 1) were kindly provided by Emisphere Technologies, Hawthorne, NY. Krebs-Ringer Buffer (KRB), verapamil, progesterone, and myristoylcarnitine were obtained from Sigma Chemical, St. Louis, MO. Oleic acid was purchased from Fisher Scientific, St. Louis, MO. DPH and TMA-DPH were purchased from Molecular Probes (Eugene, OR), and the CytoTox96® kit was obtained from Promega (Madison, WI).

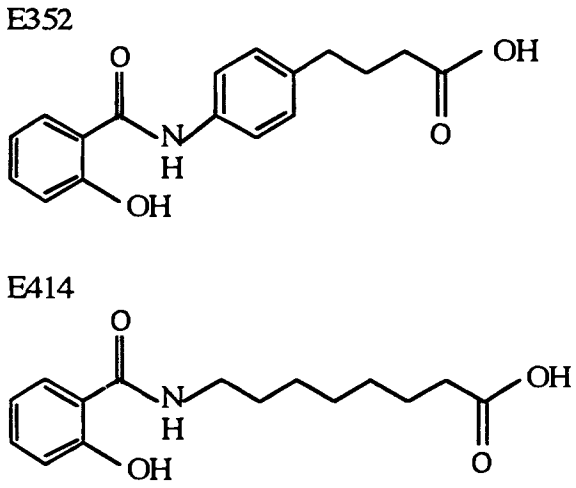

Fig. 1. Chemical structure of the novel enhancers.

Cell Culture

Caco-2 cells, a human colon carcinoma cell line, were maintained in Dulbecco's Modified Eagle medium (DMEM) containing 10% fetal bovine serum, 1% non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells of passage number 25–50 were used. The medium was changed every other day. The cells were expanded in tissue culture flasks (75 cm^2 growth area) at 37°C in 90% relative humidity and 5% CO_2 until confluence. The cell were detached from the flasks by washing with 0.2% EDTA in phosphate-buffered saline (PBS), followed by 0.5% trypsin and 0.2% EDTA in PBS. The cell suspensions were added with concentration of 2×10^5 cells/cm^2 onto polycarbonate membrane Snapwell® culture plate inserts pretreated with collagen. The cell monolayers were used between 21–28 days after seeding.

Transport Study

The inserts containing Caco-2 cell monolayers were placed between diffusion chambers, which were maintained at a constant temperature of 37°C, unless stated otherwise, by a water-heated jacket and 5 ml of transport medium, KRB, pH 7.8, was placed in each of the donor and receiver compartments. Mixing and aeration in the diffusion cell was achieved by a 5% CO_2/95% O_2 airlift. One ml of sample was taken from the receiver side at each time point and replaced with an equal volume of transport medium. The duration of each experiment was 120 minutes. The permeabilities in both apical to basolateral (AP to BL) and basolateral to apical (BL to AP) directions were determined.

Transport studies conducted in the presence of the P-gp-like efflux system inhibitors, i.e., verapamil (300 μM) and progesterone (100 μM), were carried out essentially as above with minor modifications. The cell monolayers were first preincubated with the inhibitor solutions for 30 minutes. The same concentration of inhibitors was also present in both apical and

basolateral compartments at all times during the transport studies. In a control study, data not shown, we monitored the effect of both verapamil and progesterone on [^{14}C]-mannitol flux across Caco-2 monolayers. Both verapamil and progesterone did not significantly change the flux of mannitol within two hours. The radioactivity of each sample was measured on a Beckmann LSC 6000IC (Beckmann Instruments, Arlington Heights, IL). The permeability coefficient (P) was calculated using the following equation:

$$P = \frac{V}{A \cdot Co} \cdot \frac{dC}{dt} \quad (1)$$

where dC/dt is the flux across the monolayer, V is the volume of the chamber (5 ml), A is the surface area (1 cm^2), and C_0 the initial concentration in the donor compartment. All data were analyzed and statistical differences between two means were evaluated using Student's *t* test.

Fluorescence Anisotropy Measurement

Caco-2 cells were grown to confluence in 25 cm^2 cell culture flasks. Confluent monolayers were washed three times with phosphate buffer (PBS, pH 7.4) and incubated with novel enhancers or KRB solutions for 0, 30, 60, and 120 minutes at 37°C. After three washing with PBS, the cells were trypsinized and resuspended in PBS to a final concentration of 2×10^5 cells/ml. The Caco-2 cell suspensions were labeled with DPH by adding 2.5 ml of 1 mM freshly prepared DPH stock solution in tetrahydrofuran to 2.5 ml of cell suspension. Suspensions were then incubated at room temperature in the dark for 30 minutes to complete labeling. Similarly, cells were labeled with TMA-DPH by adding 2.5 ml of 1 mM TMA-DPH stock solution in dimethylformamide to 2.5 ml of cell suspension. The TMA-DPH labeled suspensions were incubated at room temperature in the dark for 2 minutes before measurement.

The fluorescence anisotropy of DPH or TMA-DPH labeled Caco-2 cells was measured with an I.S.S. KOALA fluorometer (Urbana-Champaign, IL) in an L-format, excited with 360 nm vertically polarized light and measured emission intensity at 430 nm in the direction parallel and perpendicular to the exciting light. The steady-state fluorescent anisotropy is calculated with the following equation:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (2)$$

where r is anisotropy, I_{VV} and I_{VH} are the intensity measured in directions parallel and perpendicular to the polarized exciting light, respectively, and G was calculated as I_{HV}/I_{HH}.

Lactate Dehydrogenase Assay

The amount of lactate dehydrogenase (LDH) released from Caco-2 cells after treatment was assayed using CytoTox96® obtained from Promega (Madison, WI). LDH is a cytosolic enzyme that is normally not secreted outside of the cell. However, upon cell membrane damage, it is released into the culture medium. Released LDH in culture medium is measured with a 30-minute coupled enzymatic assay which results in the conversion of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) into a red formazan product. The amount of color formed is proportional to the amount of LDH released into the culture medium. Briefly, the monolayers were cultured in a 12-well transwells and maintained as described earlier. The cells between 21–28 days were washed three times with PBS, then treated with KRB, enhancer, or 1% Triton X-100 solutions in the presence of hGH for 0, 30, 60 and 120 minutes, incubated in a 90% humidity, and 5% CO_2 chamber at 37°C. Fifty μl of solution from the donor and receiver chambers were collected and diluted with 50 μl KRB. Fifty μl of this aliquot was transferred into a fresh 96 well flat bottom plate and another 50 μl of reconstituted substrate mix, containing diaphorase, lactate, NAD^+ and INT, was added. The plate was covered and incubated for 30 minutes at room temperature. The absorbance was read at 490 nm using a multiwell scanning spectrophotometer (EL-312e, Bio-Tek Instruments, Winooski, VT).

RESULTS

P-gp-Like Efflux System Involvement

It was observed that the effect of these novel enhancers on hGH absorption through Caco-2 cells is asymmetric (Fig. 2) where there is a higher permeation rate in the BL to AP direction, and therefore, it was speculated that P-glycoprotein-like efflux systems are involved in the enhancement mechanism of these novel enhancers. We established that Caco-2 cell monolayers grown in our laboratory express P-gp-like efflux systems using a known substrate-vinblastine, and competitive inhibitors-verapamil and progesterone. It was demonstrated (data not shown) that vinblastine did show a lower permeation rate from the mucosal to serosal side compared with that from the serosal to mucosal side, which suggested that there are P-gp-like efflux systems in the apical surface of Caco-2 monolayers that pumped vinblastine back out to the mucosal side, as suggested by several other authors (14). On the other hand, when competitive inhibitors, i.e., verapamil and progesterone, were applied to the system, the unequal fluxes of vinblastine disappeared suggesting

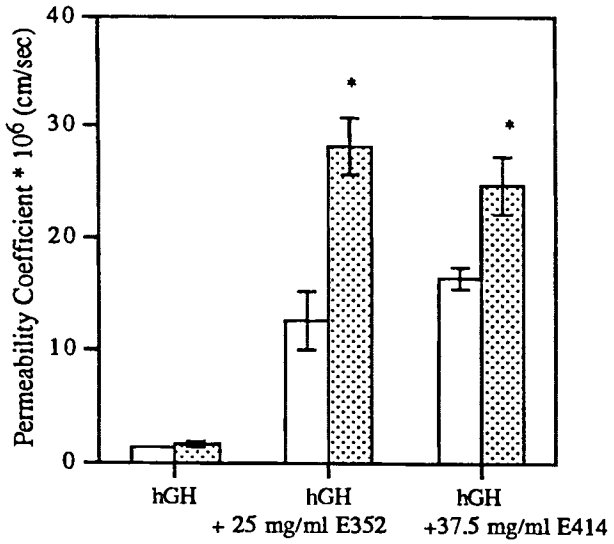

Fig. 2. Transport of [^{125}I]-hGH across Caco-2 cell monolayers in the absence or presence of novel enhancers. (□) represents the transport in the apical to basolateral direction, and (▩) represents the transport of basolateral to apical direction. Each value was an average ± s.e.m.; n = 3–9. * Denotes a significant difference of $p < 0.05$ between apical to basolateral and basolateral to apical direction.

that these two drugs successfully blocked the P-gp-like efflux systems.

It was also shown (Fig. 3) that novel enhancers, E352 and E414, both are substrates for P-gp-like efflux systems since the unequal fluxes of vinblastine again disappeared similar to that of verapamil and progesterone, when novel enhancers were applied. This further suggested that novel enhancers themselves are P-gp substrates. When hGH and novel enhancers were tested with competitive inhibitors-verapamil and progesterone, the unequal fluxes seen with hGH and novel enhancers alone again disappeared. This can be explained in that verapamil and progesterone interact with P-gps first and block further transport of hGH-novel enhancer complexes through P-gps. This provides evidence that the hGH-novel enhancer complexes are substrates for P-gps (Fig. 4).

Since the P-glycoprotein-like efflux system is an active, energy-dependent pump, it is possible to eliminate the difference between transport directions by lowering the temperature. The transport studies were performed at 4, 15, and 25°C. As seen in Table 1, lowering the temperature to below 25°C completely eliminates the asymmetry of hGH transport in the presence of novel enhancers, thus, providing evidence for the involvement of a P-gp-like efflux system.

Membrane Fluidity

The effect of these novel enhancers on the membrane bilayer can be explored by determining membrane fluidity measurements using DPH and TMA-DPH fluorescence anisotropy. Fluorescence anisotropy is inversely related to membrane fluidity, therefore, if there is a decrease in DPH fluorescence anisotropy upon novel enhancer exposure, it may be concluded that there is an increase in membrane fluidity within the lower portions of the cell bilayer. The same may be concluded with TMA-DPH, except that its location of greater fluidity is within the upper surface of the bilayer. The overall consequence would be that the membrane structure has been altered which may lead to subsequent enhancement of drug absorption.

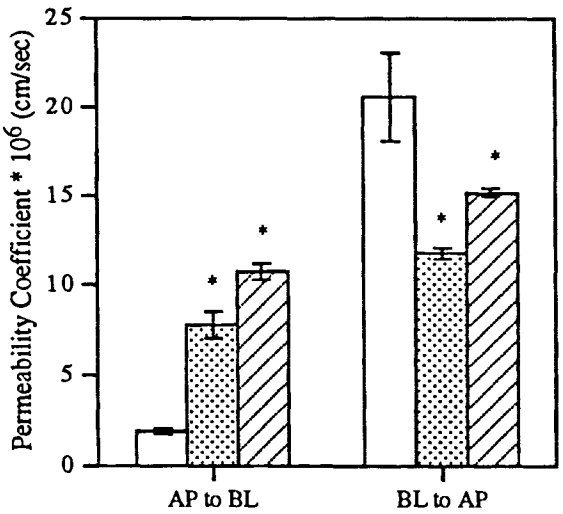

Fig. 3. Effect of novel enhancers on transport of the P-glycoprotein substrate [^{3}H]-vinblastine across Caco-2 cell monolayers. (□) represents vinblastine alone in buffer, (▩) represents vinblastine with 25 mg/ml E352, and (▨) represents vinblastine with 37.5 mg/ml E414. Each value was an average ± s.e.m.; n = 3–9. * Denotes a significant difference of $p < 0.05$ compared with same condition in buffer.

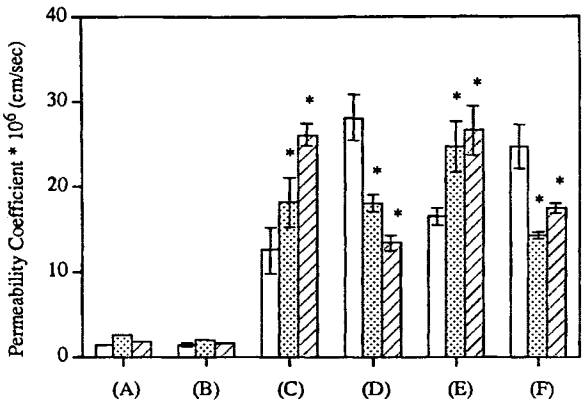

Fig. 4. Effect of P-glycoprotein inhibitors on transport of [^{125}I]-hGH across Caco-2 cell monolayers. (A) hGH alone, AP to BL direction; (B) hGH alone, BL to AP direction; (C) hGH in the presence of 25 mg/ml E352, AP to BL direction; (D) hGH in the presence of 25 mg/ml E352, BL to AP direction; (E) hGH in the presence of 37.5 mg/ml E414, AP to BL direction; and (F) hGH in the presence of 37.5 mg/ml E414, BL to AP direction; (□) represents the condition in buffer alone, (▩) represents the condition with 300 μM verapamil as an inhibitor, and (▨) represents the condition with 100 μM progesterone as an inhibitor. Each value was an average ± s.e.m.; n = 3–9. * Denotes a significant difference of $p < 0.05$ compared with same condition in buffer.

The *r* value for the controls of DPH and TMA-DPH labeled Caco-2 cell membrane at zero time point were about 0.187 ± 0.000, and 0.287 ± 0.001, respectively. Relative to control, other known enhancers (i.e., 1 mM oleic acid and 0.1 mM myristoylcarnitine) showed a significant decrease in anisotropy of both DPH and TMA-DPH (Fig 5) both in the presence and absence of 50 nM of hGH. On the other hand, the fluorescence anisotropy of both DPH and TMA-DPH did not show substantial changes induced by E352 or E414 alone, but when 50 nM hGH coexisted with these novel enhancers, the anisotropy of both DPH and TMA-DPH were much lower than that of the

Table 1. Effect of Temperature on the Transport of [^{125}I]-hGH Across Caco-2 Cell Monolayers

	Temperature (°C)	AP to BL	BL to AP
hGH	4	0.18 ± 0.05	0.14 ± 0.07
	15	0.28 ± 0.04	0.27 ± 0.05
	25	0.48 ± 0.07	0.41 ± 0.08
	37	1.34 ± 0.19	1.45 ± 0.24
hGH	4	14.27 ± 1.21	11.10 ± 1.25
+ 25mg/ml	15	16.87 ± 1.79	17.31 ± 0.66
E352	25	13.58 ± 0.60	16.42 ± 0.22
	37	12.58 ± 2.68	28.10 ± 2.61*
hGH	4	15.60 ± 1.17	15.75 ± 1.39
+ 37.5mg/ml	15	14.12 ± 1.36	14.13 ± 0.93
E414	25	11.52 ± 1.77	14.50 ± 0.20
	37	16.34 ± 0.97	24.66 ± 2.64*

Note: Each value was an average ± s.e.m.; n = 3–9. * denotes a significant difference of $p < 0.05$ between apical to basolateral and basolateral to apical direction.

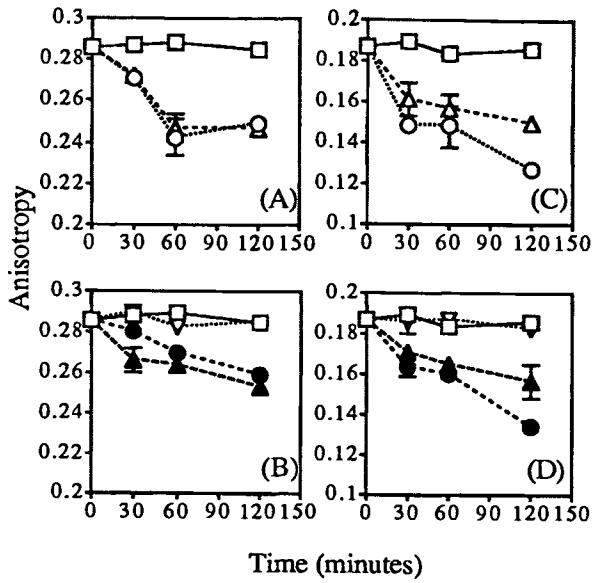

Fig. 5. Effect of classical absorption enhancers on the changes of fluorescence anisotropy in Caco-2 cells. (A) & (B) represent the changes of TMA-DPH anisotropy, and (C) & (D) represent the changes of DPH anisotropy; (□) Krebs-Ringer Buffer, (○) 1 mM oleic acid, (△) 0.1 mM myristoylcarnitine, (▽) 50 nM hGH in Krebs-Ringer Buffer, (●) 50 nM hGH + 1 mM oleic acid, and (▲) 50 nM hGH + 0.1 mM myristoylcarnitine. Each value was an average ± s.e.m.; n = 3–6.

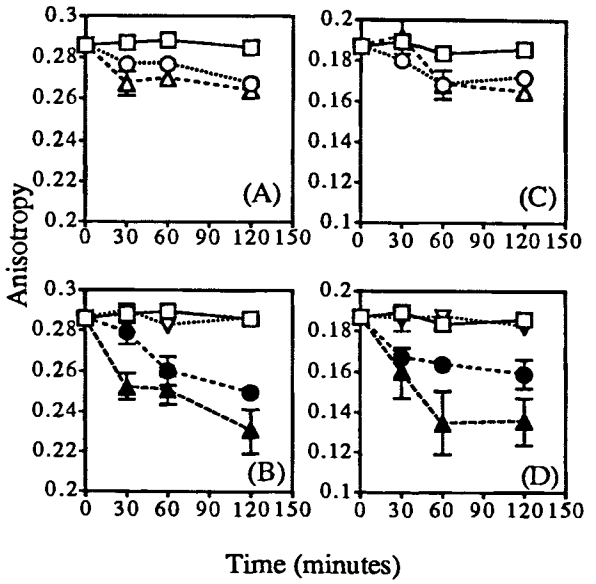

Fig. 6. Effect of novel enhancers on the changes of fluorescence anisotropy in Caco-2 cells. (A) & (B) represent the changes of TMA-DPH anisotropy, and (C) & (D) represent the changes of DPH anisotropy; (□) Krebs-Ringer Buffer, (○) 37.5 mg/ml E414, (△) 25 mg/ml E352, (▽) 50 nM hGH in Krebs-Ringer Buffer, (●) 50 nM hGH + 37.5 mg/ml E414, and (▲) 50 nM hGH + 25 mg/ml E352. Each value was an average ± s.e.m.; n = 3–6.

novel enhancer alone, and were in about the same range as that of oleic acid and myristoylcarnitine, as shown in Fig. 6.

LDH Assay

The release of biochemical damage markers into the bathing medium was studied using CytoTox96® kits. Figure 7 demonstrated that both E352 and E414 at their effective concentrations in the presence of 50 nM hGH, caused negligible amounts of LDH to be released into the bathing medium up to 1 hour, which corresponds well with membrane fluidity measurements. However, we did observe a raise in LDH leakage after 2 hour treatment which suggested some membrane damage induced by these novel enhancers at long contact time.

DISCUSSION

The pathway of permeation of a solute is determined by its physicochemical properties and the barrier characteristics of the epithelium. Due to the hydrophilicity and large size of proteins, it is generally believed that there are only two major transport mechanisms involved in most peptide and protein absorption across normal intestinal epithelium, i.e., pino/endocytosis and passive diffusion through the intercellular space (paracellular pathway). However, it is not impossible for these macromolecules to cross biological membrane directly. Recently, the three-dimensional conformation of proteins has been demonstrated to potentially affect absorption of protein drugs (15). For example, it has been reported that some proteins are translocated through cell membranes in a loose conformation (16), i.e., molten globular state, which is more hydrophobic than the native form. Furthermore, it was also found that negatively charged lipids could interact with a partially unfolded (i.e., molten globular) acid fibroblast growth factor which may play a role in membrane translocation of that protein (17). A current report by Johnston *et al.* (15) also demonstrated that the increase in basic fibroblast growth factor (bFGF) permeation through rabbit buccal epithelium can be achieved by first denaturing the protein with guanidine (GnHCl) followed by exposure to a permeation enhancer, sodium glycocholate. It was shown that GnHCl caused unfolding of bFGF which resulted in a more linear, smaller dimension of bFGF. Once bFGF has been unfolded, addition of the permeation enhancer augments transport of the more linear, higher energy unfolded form of bFGF across the buccal mucosa. Therefore, we hypothesized that the mechanism of these novel enhancers is to preferentially interact and stabilize partially unfolded human growth hormone which is more hydrophobic, which can be translocated through plasma membranes easier.

If the hypothesis is correct, the more hydrophobic form of hGH, in the presence of these novel enhancers, should translocate through the membrane directly. The above studies provide evidence that the transport of hGH in the presence of these novel enhancers across Caco-2 cell monolayers is affected by a polarized pump operating in the BL to AP direction. Furthermore, by utilizing specific inhibitors of the P-glycoprotein-like pumps, we demonstrated that a P-glycoprotein-like transporter is involved in the enhancement mechanism of these novel enhancers. Since the only common characteristic between P-glycoprotein substrates is hydrophobicity, it is reasonable to suggest that these novel enhancers interact with hGH to make hGH more hydrophobic. Therefore, hGH can be affected by a P-glycoprotein-like transporter in the presence of these novel enhancers but not alone. Furthermore, since the P-glycoprotein substrate binding site is believed to locate inside the apical

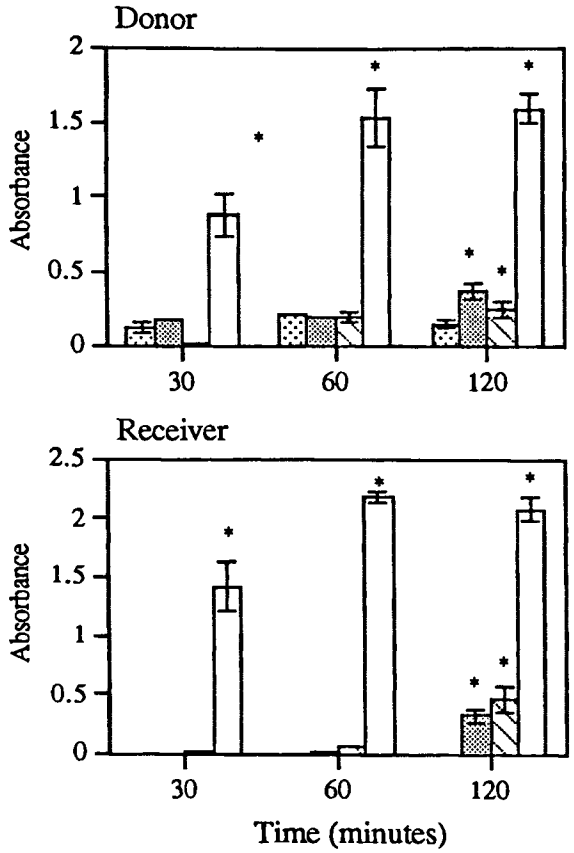

Fig. 7. The lactate dehydrogenase (LDH) activity measured by absorbance at 490 nm from either donor or receiver solution with or without novel enhancers. Graph A is the enzyme activity in the donor solution, and B is in the receiver solution. (▩) represents cells treated with 50 nM hGH in KRB, (▦) represents cells treated with 50 nM hGH + 25 mg/ml E352, (▧) represents cells treated with 50 nM hGH + 37.5 mg/ml E414, and (□) represents cells treated with hGH + 1% Triton X-100. Each value was an average of 3–6 repetitions. * Denotes a significant difference of $p < 0.05$ compared with control.

membrane and we assume the present transporter, if not a P-glycoprotein, is also located inside the apical membrane, the substrates have to be able to partition into the lipid bilayer or the cytoplasm to be recognized and pumped out by the P-glycoprotein. This further suggests that hGH is transported through membrane lipid bilayers of Caco-2 cells in the presence of these novel enhancers.

However, there are other possible explanations for the higher rate of permeation in the serosal to mucosal direction since these novel enhancers are themselves substrates of P-gps. It could be argued that the concentration of the novel enhancers was higher at the mucosal side when the novel enhancer was applied to the mucosal side because of the back efflux by P-gps. In other words, the amount of novel enhancers at the serosal side was lower. Due to their surfactant-like properties, the higher the novel enhancer concentrations, the more perturbation of cell membranes. Therefore, the concentration of novel enhancers at the serosal side of cell membranes was lower with less perturbation to the serosal membrane and thus the permeation rate of hGH was lower from the mucosal to serosal side. On the other hand, when novel enhancers were applied to the serosal side, the mucosal side of enhancer concentration became higher due to the efflux by P-gps. This caused more perturbation of the membrane and a higher permeation rate for hGH from the serosal to mucosal side. If this is the case, when novel enhancers were applied to both sides, in the same concentration, which could perturb the cell membrane to the same degree, the permeation rate in these two direction should be the same, and much higher than the rate when novel enhancers were applied only to one side. Also, the temperature should not have a significant effect on the permeation rate since the passive diffusion process is not strongly temperature-dependent. However, it was demonstrated (data not shown) that the unequal fluxes disappeared when these novel enhancers were present on both sides, but the rate was in between those when they are present only on one side, not higher. Furthermore, it was shown that the unequal fluxes were eliminated by lowering temperature to 25°C or lower, suggesting that the difference between the two fluxes was highly energy-dependent. Therefore, the effect of unequal fluxes of hGH in the presence of novel enhancers is not due to their surfactant-like properties but the effluxers.

A more direct measure to determine the transmembrane transport mechanism of these novel enhancers is to measure disruption of the basic structure of lipid packing order of cell membranes. Fluorescence polarization is a sensitive technique for the study of fluidity or the lipid packing order of membranes. DPH and TMA-DPH are the most popular pair of probes used in the study of membrane fluidity. DPH is a marker for fluidity in the core of the cell membrane, whereas its cationic analog, TMA-DPH, is anchored at the surface of the plasma membrane and reflects the state of the superficial membrane lipids. The fluorescence anisotropy of these probes increases when lipid packing becomes more ordered or rigid; alternatively fluorescence anisotropy decreases when the membrane becomes more disordered or random. Several permeability enhancers were shown to change membrane fluidity. Benzyl alcohol decreased polarization of DPH fluorescence in erythrocyte membrane (18), and similarly, oleic acid (19) and myristoylcarnitine were shown to affect membrane fluidity of the epithelial membrane lipid domains (20). Similarly, some proteins which possess the ability to cross cell membranes directly, such as magainin (21), mellitin peptide (22), and diphtheria toxin fragment B (23), caused an almost instantaneous perturbation to the cell membrane by deeply inserting themselves into the membranes.

The fluorescence anisotropy of both DPH (~5–7%) and TMA-DPH (~7–11%) was decreased slightly in the presence of novel enhancers after two hours of treatment, while the anisotropy of DPH and TMA-DPH was decreased (~13% and 20–32%, respectively) in the presence of other known enhancers (i.e., 1 mM oleic acid and 0.1 mM myristoylcarnitine). The data suggested that novel enhancers themselves do not cause significant damage to cell membranes. On the other hand, when hGH and novel enhancers coexist in solution, the anisotropy of both TMA-DPH an DPH were much lower than that of hGH or novel enhancers alone and about the same magnitude 1 mM oleic acid and 0.1 mM myristoylcarnitine. This suggested that novel enhancers interacted with hGH and the membrane perturbation ability of the complex is higher than that of novel enhancers or hGH alone.

Lastly, the safety issue of using these novel enhancers as absorption enhancers was also considered. LDH is a cytosolic

enzyme, and its presence in the apical compartment is generally regarded as evidence for cell membrane damage. As demonstrated in the result section, it is observed that novel enhancers cause no membrane damage compared with controls within one hour. This is also consistent with membrane fluidity measurements of novel enhancers alone. However, after two hour incubation, there is an increase in release of LDH suggesting that novel enhancers can cause some cell membrane damage when treated for extended periods of time.

In conclusion, the recognition of P-gp-like transporter of these complexes suggests that these complexes are indeed more hydrophobic than hGH itself. Furthermore, the fluidity studies support that the complex actually passively diffuse through cell plasma membrane which causes a significant increase in membrane fluidity. The data above indirectly support the premise that the novel enhancer-induced hGH absorption is by interacting with hGH specifically to presumably make a more hydrophobic, more linear and smaller dimension form of hGH, e.g., the unfolded state. Since the complex is more hydrophobic and with a smaller dimension, the passive diffusion rate of the complex through a lipid bilayer is higher.

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