Sublingual Delivery of Insulin: Effects of Enhancers on the Mucosal Lipid Fluidity and Protein Conformation, Transport, and in Vivo Hypoglycemic Activity

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The purposes of this study were to evaluate effects of enhancers for sublingual delivering insulin on the mucosal lipid fluidity and protein conformation, transport, and in vivo hypoglycemic activity in normal rats. The effects on sublingual mucosa, and aggregation states of insulin were estimated using fluorescence polarization, and circular dichroism method, respectively. The human immortalized oral epithelial cell monolayer was used for evaluating transport of insulin. Hydroxypropyl-β-cyclodextrin (HP-β-CD), chitosan, polyethylene–polypropylene glycol, polyoxylethylene lauryl ether, polysorbate 80, egg lecithin, or oleic acid, was used as a penetration enhancer, respectively. The fluidity of sublingual mucosal lipid was markedly reduced by these enhancers excluding polysorbate 80, and the secondary structure of the mucosal proteins was also influenced by these enhancers. The hexamers of insulin were dissociated to monomers only by chitosan, polyoxylethylene lauryl ether, and egg lecithin. Nonetheless, plasma glucose levels in normal rats were significantly lowered after sublingual administration of insulin with an enhancer compared with those without an enhancer at the same time-point. The enhancing effects may be due to one or multiple factors: increasing the mucosal lipid fluidity, directly loosing the tight junction of epithelia, and dissociating the hexamers of insulin to monomers. Among these, the opened tight junction may correlate most with the enhancing effect in the mucosal permeability. Because the aggregates of insulin exist, the dissociation of the aggregates by an enhancer would benefit the permeability.

Key words insulin; enhancing mechanism; transport; hypoglycemic activity; sublingual delivery

The intensive insulin therapy regimens involving multiple daily subcutaneous injections place a heavy burden of compliance on diabetic patients. Therefore, the nasal, oral, gastrointestinal, and transdermal administration routes have been investigated as alternatives for less invasive routes of delivery in spite of current unsuccessful exploit.1 Only a limited number of polypeptides (e.g. insulin, oxytocin, and vasopressin) have been exploited as therapeutics because of problems related to their delivery. The sublingual mucosa offers an alternative route to the parenteral administration. Peptides are generally not well absorbed through mucosa due to their molecular size, hydrophilicity and the low permeability of the membrane. Therefore, peptide preparation for nonparenteral administration needs to be formulated with penetration enhancers. Transmucosal administration of insulin has been reported since early 1980’s.2,3 Buccal absorption of insulin was studied in beagle dogs by Ritschel et al. (1989),3 and their results showed that the buccal absorption extent of insulin was increased to 18.3%. In contrast, the oral absorption of insulin through gastrointestinal (GI) tract was estimated below 5% due to the metabolism effects in the GI tract. Two possible pathways for drug absorption have been identified: transcellular and paracellular pathways. The transcellular pathway is generally a principal route for drugs with some degree of lipophilicite. Because of less lipophilicity, peptides are reported to be permeated through the aqueous pathways by passive diffusion, including the paracellular across the junctional complexes and aqueous pore path which probably play significant roles in the delivery of peptide drugs.4–7 Peptide transport across oral mucosa occurs via passive diffusion and is often accompanied by varying degrees of metabolism. Various strategies to improve the oral absorption of peptides were suggested including the use of penetration enhancers to increase membrane permeability and/or the addition of enzyme inhibitors to increase their stability, modification with bioreversible chemical groups or specific formulations like bioadhesive delivery systems.8,9 For a peptide molecule, passage across an organized mucosal passage of peptide molecules through the paracellular pathway is restricted by the mucosal tight junction. One approach to overcoming this barrier is to sublingually coadminister peptides with absorption enhancers. When the enhancers are included the formulation of peptide for nonparenteral administration, they may alter the structural and functional features of cell membrane or peptide itself: fluidity, conformation of protein in mucosal membrane, cell tight junctions, and structure of active peptide. During the past 20 years, a great deal has been published on the subject of peptide absorption. Despite this, the mechanism for sublingual absorption of insulin in presence of various enhancers remained unknown.

Proteins in the mucosal membrane are involved in the ones inserted in the bilayers of cells and those for linking the gap junctions. Three types of junctions, namely, gap junctions, tight junctions and adherent junction, have been discovered in endothelial cells.10,11 Gap junctions are clusters of transmembrane hydrophilic channels that allow for direct exchange of ions and small molecules between adjacent cells. The role of adherent junctions in the regulation of permeability has been recently discovered,12,13 and vascular endothelial cadherin, alpha-catenin, beta-catenin, and plakoglobin are involved in the adherent junction of vascular endothelial cells.
Tight junctions play a crucial role in permeability modulation, and they are formed primarily by a family of transmembrane proteins and occludin is the most studied.\(^{14}\) Other peripherally localized cytoplasmic proteins such as ZO-1,\(^{15}\) ZO-2 (160-kDa polypeptide which binds to ZO-1),\(^{16}\) and claudin,\(^{17}\) 7H6 antigen,\(^{18}\) and symplekin\(^{19}\) appear to link occludin with the actin-based cytoskeleton. The importance of tight junctions has been shown in the brain-blood barrier where brain endothelial cells with high levels of occludin expression had remarkably low permeability. The proteins localized in the sublingual cell membrane and related to tight junction have not been fully characterized although the claudins, occludin,\(^{20}\) and junction-enriched -associated protein (JEAP)\(^{21}\) may be involved in this tight junction.

To understand the effects of enhancers on the lipid fluidity of sublingual mucosa, fluorescence polarization is included in the present study. The theory of fluorescence polarization, first described in 1926 by Perrin (http://www.tecan.com/fp_introduction1.pdf), is based on the observation that fluorescent molecules in solution, excited with plane-polarized light, will emit light back into a fixed plane (i.e. the light remains polarized) if the molecules remain stationary during the excitation of the fluorophore. Molecules, however, rotate and tumble, and the planes into which light is emitted can be very different from the plane used for initial excitation. 1,6-Diphenyl-1,3,5-hexatriene (DPH), an oblate and flat molecule which is used as fluorescent probe, can be inserted into the bilayer and arranged in a parallel direction with hydrocarbon chains of lipid membrane. If movement activity of the lipid chains is increased, DPH molecules would swing with the lipid chains resulting in a decreased the fluorescence polarizability (\(P\)) of DPH and a decreased microviscosity (\(\eta\)) of cell membrane. Therefore, the polarizability and microviscosity reflect the fluidity alteration of lipid membrane. To evaluate the effects of enhancers on the secondary structures of sublingual mucosal proteins, and the aggregation states of insulin, the circular dichroism method was used.

The purposes of this study were to evaluate the effects of enhancers for sublingual delivering insulin on the mucosal lipid fluidity and protein conformation, transport pathway, and in vivo hypoglycemic activity in normal rats.

**MATERIALS AND METHODS**

**Materials and Animals** Human immortalized oral epithelial cells (HIOEC) transfected with human papillomavirus (HPV16 E6/E7) open reading frames using recombinant retroviral system (pLXSN) was kindly provided Prof. Wan-Tao Chen (Shanghai Second Medical University, Shanghai, China).\(^{22,23}\) Epilife medium added with human keratinocyte growth supplement (HKGS) was purchased from Cascade Biologics (Portland, OR, U.S.A.). Hanks’ balanced salt solution without phenol red (HBBS) was from Beijing Chemical Reagent Company (Beijing, China). Costar 12-well transwell, filter, polycarbonate membranes, and 96-well culture plates were purchased from Corning (Corning, NY, U.S.A.); epithelial volthometer (EVOM) was from World Precision Instruments Inc. (Sarasota, FL, U.S.A.); horizontal orbital plate shaker was from Herbin Donglian Electronics (Herbin, China); \(^{125}\)I-insulin radioimmunoassay kit was from Atomic Academy of China (Beijing, China); microplate reader (Bio-Rad Model 550) was from Bio-Rad Laboratories (Hercules, CA, U.S.A.).

The chemicals and suppliers were: crystalline porcine zinc insulin (27.7 IU/mg), Xuzhou Biochemical Plant (Xuzhou, China); polyoxyethylene lauryl ether (brij 35), \(\beta\)-lucine, colchicine, and poly-L-lysine, Beijing Chemical Plant (Beijing, China); hydroxypropyl-\(\beta\)-cycloexdirinx (HP-\(\beta\)-CD), Shanxi Liquan Chemical Corporation (Taiyuan, China); chitosan (non-salt form), Qingdao Haihui Chemical Plant (Qingdao, China); egg lecithin, Shanghai Taiwei Co. Ltd. (Shanghai, China); polyethylene–polypropyylene glycol (poloxamer 188), and polysorbate 80 (tween 80), Beijing Tianrun Shanda Biotech Co., Ltd. (local Sigma and GIBCO products agent, Beijing, China). All other chemicals and solvents were of analytical grade.

Male Sprague–Dawley rats were obtained from Experimental Animal Center of Peking University. All care and handling of animals were performed with the approval of Institutional Authority for Laboratory Animal Care.

**Preparation of the Homogenates of Sublingual Mucosa**

Normal male Sprague–Dawley rats weighing 210—240 g were immediately sacrificed after being anesthetized with ether and the sublingual membrane was removed, as described by Kinnaman et al.\(^{24}\) The membrane slices were washed for 3 times using cold D-Hanks solution, and wiped off. The membrane slice was cut into chips using a pair of scissors, washed using cold D-Hanks solution. The sample was then homogenized in cold physiological saline (mucosa: saline = 1:3, w/w) at 4°C. The homogenate was filtered using the 3-layer sterilized gauze and the filtrate was centrifuged (300 revolution per min) for 1 h at 4°C. The supernatant was collected, slightly homogenized at 4°C, sonicated for 1 min, and centrifuged (30000 revolution per min) for 1 h. The pellets were collected, and weighed as the homogenates of sublingual mucosa.

**Effect on the Fluidity of Sublingual Lipid Mucosa**

HP-\(\beta\)-CD (10%, w/v), polyeethylene–polypropylene glycol (10%, w/v), polyethylene–laurylether (10%, w/v), and polyoxyethylene lauryl ether (10%, w/v) were prepared at 37°C water bath using PBS (10 mmol/l potassium dihydrogen phosphate, and 40 mmol/l sodium hydroxide, pH 7.4), respectively. Chitosan solution (10%, w/v) was prepared using pH 3.0 PBS (adjusted the PBS to pH 3.0 using diluted HCl solution). Oleic acid (10%, w/v), and egg lecithin solution (10%, w/v) prepared using propylene glycol, respectively. These solutions were named as enhancer solutions below.

An aliquot of 0.5 ml homogenates of sublingual mucosa (10 mg/ml) was added with equal volume of an enhancer solution, respectively, and incubated at 37°C for 30 min, and then labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH) at 37°C for 30 min. The final concentration of DPH in the homogenates was 1 \(\mu\)mol/l. The polarizability (\(P\)) and microviscosity (\(\eta\)) values of the homogenate lipid were measured using Toshiba F-3010 fluorescence spectrophotometer (Tokyo, Japan). The excitation wavelength of the fluorescence was set at 362 nm and the emission wavelength was 432 nm. The scanning speed was 60 nm/min and the slit was 20 nm.

**Effects on the Protein Conformation of Sublingual Mucosa**

An aliquot of 0.5 ml homogenates of sublingual mucosa (10 mg/ml) was added with equal volume of an en-
hancer solution, and mixed homogenously, respectively. The blend of an enhancer with homogenates was incubated at 37 °C for 30 min, filled in a 1 mm path-length quartz cuvette, and measured using a Jasco-715 circular dichroism spectropolarimeter (Tokyo, Japan) with a scan rate of 50 mm/min at room temperature. The spectrum was recorded in the range of 190—250 nm, and analysis for secondary structure was performed with the aid of Jasco J-700 software (Tokyo, Japan).

**Effects on the Association Characteristics of Insulin**

Insulin dissociation states changed from hexameric to monomeric forms in the presence of various enhancers were evaluated using a Jasco-715 circular dichroism spectropolarimeter. An appropriate amount of insulin was dissolved in PBS (pH 7.4) at 37 °C for preparing 0.5, and 1.0 mg/ml of insulin solution, respectively. Aliquots of 0.5 ml insulin solutions (1.0 mg/ml) were mixed with 0.5 ml HP-β-CD (10%, w/v), polyethylene–polypropylene glycol (10%, w/v), polysorbate 80 (10%, w/v), oleic acid (10%, w/v), polyoxyethylene lauryl ether (10%, w/v), chitosan solution (10%, w/v), and egg lecithin solution (10%, w/v), respectively. Insulin (0.5 mg/ml) PBS solution, or insulin (0.5 mg/ml) solution containing an enhancer (5%, w/v) was scanned from 250 to 300 nm at a scanning speed of 100 nm/min at 25 °C, respectively. A 10 mm path-length quartz cuvette was used to obtain optimum resolution of the circular dichroism spectra. The molecular ellipticities were measured and the spectra recorded, as described by Shao et al.25 and Uchiyama et al.26

**Effects on the Transport of Insulin across HIOEC Monolayer**

HIOEC cells were cultured for 2 weeks in Epilife medium (pH 7.4) at 37 °C incubator with an atmosphere of 5% CO2/95% air (relative humidity, 98%). Cell subcultivations were performed by trypsinization using 0.25% (w/v) trypsin and 0.02% (w/v) EDTA, and extruded through polyethylene–polypropylene glycol (10%, w/v), 5% egg lecithin or 5% oleic acid, w/v) was subjected into the rat of Group I as a positive control. The insulin solution (1 IU/kg) or insulin solution (10 IU/kg) containing a kind of penetration enhancer (5% HP-β-CD, 5% chitosan, 5% polyethylene–polypropylene glycol, 5% polysorbate 80, 5% egg lecithin or 5% oleic acid, w/v) was sublingually administered by spraying to each rat of ‘Group II’ through ‘Group IX’, respectively. A 200–µl blood specimen was collected from orbital vein at 0, 15, 30, 60, 90, 120, 180, 240 min using a heparinized capillary tube, and centrifuged at a speed of 3000 revolution per minute for collection of plasma. Plasma glucose levels were measured using the 125I-insulin radioimmunoassay kit.

**Calculation and Statistical Analysis**

The parameters of secondary structure of mucosal proteins, viz., α-helix (%), β-sheet (%), β-turn (%), random coil (%) were obtained by simulating analysis from the Jasco J-700 software. Apical to basolateral apparent permeability (Papp) values of insulin across HIOEC monolayer were calculated with the equation according to Violante (2004), $P_{app} = \frac{\Delta Q}{\Delta t}$, $A$, and $C_i$ is the initial concentration of insulin in the donor compartment.

In order to compare the glucose-lowering effects of various treatments, the difference value of the area above the plasma glucose curve (ΔAAC) was calculated by the trapezoidal method.31 All ΔAAC values were estimated versus the glucose level after insulin was sublingually administered in absence of any enhancer.

Data are presented as the mean±S.E.M. One-way analysis of variance (ANOVA) was used to determine significance among groups, after which post-hoc tests with the Bonferroni correction were used for comparison between individual groups. A value of p<0.05 was considered to be significant.
RESULTS

Effect on the Fluidity of Sublingual Lipid Mucosa Effects of enhancers on the DPH labeled sublingual lipid mucosa are shown in Table 1. In the presence of HP-β-CD, chitosan, polyethylene–polypropylene glycol, polyoxyethylene lauryl ether, egg lecithin, or oleic acid, the polarizability and microviscosity values of the mucosal lipid were markedly reduced ($p<0.05$) compared with that in absence of an enhancer (PBS as a blank control), showing that these 6 enhancers significantly increased the fluidity of cellular membrane lipid. However, polysorbate 80 seemed not to interfere with the fluidity of cellular membrane lipid due to less reduced $P$ and $η$ values compared with that of the control. As compared with the polarizability and microviscosity values between individual groups by checking the post hoc test results, chitosan was the most significant factor which affected the fluidity of cellular membrane lipid ($p<0.01$, versus other 5 enhancers, respectively). The influencing extent was as follows: chitosan>polyethylene–polypropylene glycol>polyoxyethylene lauryl ether>egg lecithin>oleic acid>HP-β-CD ($p<0.05$, respectively).

Effects on the Protein Conformation of Sublingual Mucosa The parameters for the secondary structure of the protein conformation of sublingual mucosa are expressed as $α$-helix, $β$-sheet, $β$-turn, and random coil, as shown in Table 2. When compared with the parameters obtained from the protein treated with blank control (PBS), the changed percentage of the parameter were as follows: (1) $α$-Helix: $α$-helix percentages of the protein treated with HP-β-CD, and chitosan were increased by 26%, and 10%, respectively; those treated with polysorbate 80, and egg lecithin were decreased by 18%, and 30%, respectively. The changed $α$-helix percentages of the protein treated with polyethylene–polypropylene glycol, polyoxyethylene lauryl ether, and oleic acid were below 10%, respectively. (2) $β$-Sheet: the $β$-sheet was zero after the protein was treated with PBS buffer, HP-β-CD, polyethylene–polypropylene glycol, and polyoxyethylene lauryl ether, respectively. However, the $β$-sheet percentages of the mucosal protein treated with egg lecithin, polysorbate 80, and oleic acid were increased by 100%, 46%, and 14%, respectively. That treated with chitosan was slightly increased by 4%. (3) $β$-Turn: the $β$-turn percentages of the mucosal protein treated with HP-β-CD, egg lecithin, and polysorbate 80 were decreased by 31%, 20%, and 31%, respectively. That treated with polyoxyethylene lauryl ether was increased by 11%. However, the $β$-turn percentages of the protein treated with chitosan, polyethylene–polypropylene glycol, and oleic acid were below 10%, respectively. (4) Random coil: the random coil percentages of the protein treated with chitosan, egg lecithin, and oleic acid were reduced by 11%, 39%, and 17%, respectively. Those treated with HP-β-CD, polyethylene–polypropylene glycol, polysorbate 80, and polyoxyethylene lauryl ether were below 10%, respectively.

Effects of Enhancers on the Association Characteristics of Insulin Effects of the enhancers on the circular dichroism bands of insulin are shown in Fig. 1. The circular dichroism band of insulin treated with PBS (pH 7.4) exhibited the highest intensities of the spectra at various wavelengths, showing that insulin existed as hexamers in this buffer, in coincidence with the results of Pocker and Biswas. The negative maximal bands were attenuated on the circular dichroism spectra of insulin in the presence of egg lecithin, polyoxyethylene lauryl ether and chitosan, and these changes
in molar ellipticities indicated that the hexamers of insulin were dissociated to monomers. As the typical values, the negative absorptive intensities of insulin on the spectra at 274 nm were not significantly changed in presence of HP-β-CD, polyethylene–polypropylene glycol, polysorbate 80, and oleic acid but significantly attenuated in presence of chitosan (p<0.05), polyoxyethylene lauryl ether (p<0.05), and egg lecithin (p<0.01) as compared to that of control (PBS, pH 7.4), respectively. In view of the results from multiple comparisons between groups, the effect of egg lecithin on the association state of insulin was significantly stronger than that of polyoxyethylene lauryl ether (p<0.01), respectively. The effects of chitosan and polyoxyethylene lauryl ether were similar, as shown in Table 3.

Effects of Insulin and Enhancers on the TEER Values of HIOEC Monolayer After HIOEC monolayer was treated with 10, 50, and 100 ng/ml of insulin solutions, respectively, the TEER value of the monolayer was not significantly affected by the different insulin concentrations, as shown in Fig. 2. The TEER value was significantly reduced in presence of chitosan (p<0.01), and polyoxyethylene lauryl ether (p<0.01) as compared with that in presence of PBS (blank control) at the same time point, respectively. The effect of chitosan on the TEER values was stronger than that of polyoxyethylene lauryl ether (p<0.05). However, the TEER value was slightly changed in presence of egg lecithin, as depicted in Fig. 3.

Effects of Experimental Factors on the Insulin Transport across the Monolayer The effects of experimental factors on the insulin transport amount are depicted in Fig. 4. It was shown that the insulin transport quantities (Q, μIU) across HIOEC monolayer were not affected by the transport temperatures (37 °C or 4 °C), transport directions (AB or BA) at the same concentration (50 ng/ml), where AB represents the transport direction that is from the apical to the basolateral side, and BA is from the basolateral to the apical side. However, the transport amount at a dose of 100 ng/ml insulin was significantly higher (p<0.01) than that at a dose of 50 ng/ml insulin, showing that the transport amount of insulin was dependent of concentration characteristics.

The effects of experimental factors on the apparent permeability coefficient (P_app) of insulin across HIOEC (human im-

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**Table 3.** Insulin (0.5 mg/ml) Molar Ellipticities at 274 nm on the Circular Dichroism Spectra in Presence of Various Solutions

<table>
<thead>
<tr>
<th>Insulin solution</th>
<th>Molecular ellipticity (deg·cm²/dmol)×10⁻⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>With PBS (pH 7.4)</td>
<td>-1.01±0.11</td>
</tr>
<tr>
<td>With 5% HP-β-CD (w/v)</td>
<td>-0.96±0.05</td>
</tr>
<tr>
<td>With 5% chitosan (w/v)</td>
<td>-0.83±0.03*</td>
</tr>
<tr>
<td>With 5% polyethylene–polypropylene glycol (w/v)</td>
<td>-1.02±0.01</td>
</tr>
<tr>
<td>With 5% polysorbate 80 (w/v)</td>
<td>-0.97±0.07</td>
</tr>
<tr>
<td>With 5% polyoxyethylene lauryl ether (w/v)</td>
<td>-0.81±0.01**</td>
</tr>
<tr>
<td>With 5% egg lecithin (w/v)</td>
<td>-0.48±0.02**</td>
</tr>
<tr>
<td>With 5% oleic acid (w/v)</td>
<td>-1.05±0.02</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E.M. (n=5); *p<0.05, and **p<0.01, versus PBS; ΔΔp<0.01, versus egg lecithin, respectively.
Each TEER value was repeated for triplicates. Initial value of TEER before treatment with a penetration enhancer was 116.3±8.52 cm². TEER value % was the ratio of measured TEER value to initial one. **p<0.01, chitosan versus PBS group, *p<0.01, polyoxyethylene lauryl ether versus PBS group. *p<0.05, chitosan versus polyoxyethylene lauryl ether group at the same time point, respectively. The data are expressed as the mean±S.E.M. Results indicate that polyoxyethylene lauryl ether (5%, w/v) and chitosan (5%, w/v) significantly reduce the tight junction of the monolayer, but egg lecithin (5%, w/v) and HP-β-CD (5%, w/v) seem not to change the tight junction.

Table 4. Results showed that the $P_{app}$ values were not significantly affected by the transport temperatures (37°C or 4°C), transport directions (AB or BA), and concentrations (50 or 100 ng/ml).

**Effects of Enhancers or Additives on the Insulin Transport across the Monolayer**

Enhancers or additives were added to the apical or basolateral compartment of the monolayer at the same concentration (5%, w/v). The $P_{app}$ values were measured TEER value % was the ratio of measured TEER value to initial one. **p<0.01. The data are expressed as the mean±S.E.M., and repeated for triplicates. The results indicate that the permeation of insulin across HIOEC monolayer is significantly enhanced in presence of chitosan, egg lecithin, polyoxyethylene lauryl ether, or the additive poly-L-lysine, but slightly affected by L-leucine, or colchicine. Results indicate that the transport of insulin across HIOEC monolayer is by paracellular pathway, and dependent of tight junctional characteristics.

Table 5. The Effects of Enhancers or Additives on the Apparent Permeability Coefficient ($P_{app}$) of Insulin across HIOEC (Human Immortalized Oral Epithelial Cells) Monolayer

<table>
<thead>
<tr>
<th>Insulin concentration (ng/ml)</th>
<th>Direction</th>
<th>Temperature (°C)</th>
<th>$P_{app}$ ($\times 10^{-6}$ cm·s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>AB</td>
<td>37</td>
<td>1.95±0.58</td>
</tr>
<tr>
<td>100</td>
<td>AB</td>
<td>37</td>
<td>1.94±0.16</td>
</tr>
<tr>
<td>50</td>
<td>BA</td>
<td>37</td>
<td>1.82±0.52</td>
</tr>
<tr>
<td>50</td>
<td>AB</td>
<td>4</td>
<td>2.00±0.99</td>
</tr>
<tr>
<td>100</td>
<td>BA</td>
<td>4</td>
<td>1.76±0.07</td>
</tr>
<tr>
<td>50</td>
<td>AB</td>
<td>37</td>
<td>1.83±0.09</td>
</tr>
<tr>
<td>100</td>
<td>BA</td>
<td>37</td>
<td>1.83±0.09</td>
</tr>
<tr>
<td>50</td>
<td>AB</td>
<td>4</td>
<td>2.00±0.09</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01, versus PBS control. The data are expressed as the mean±S.E.M., and repeated for triplicates. The results indicate that the $P_{app}$ values of insulin are significantly enhanced by chitosan, egg lecithin, polyoxyethylene lauryl ether, and poly-L-lysine, respectively.

**Effects of Enhancers or Additives on the Apparent Permeability Coefficient ($P_{app}$) of Insulin across HIOEC (Human Immortalized Oral Epithelial Cells) Monolayer**

(A) The permeated insulin quantity–time profiles in presence of an enhancer, namely, 5% (w/v) of chitosan, egg lecithin, and polyoxyethylene lauryl ether, respectively; (B) the permeated insulin quantity–time profiles in presence of an additive, i.e., 10 mmol/l of L-leucine, 0.1 mmol/l of colchicine, and 1 mmol/l of poly-L-lysine. *p<0.05, and **p<0.01. The data are expressed as the mean±S.E.M., and each experiment was repeated for triplicates. These transport studies indicate that the permeated amount of insulin is significantly enhanced in presence of chitosan, egg lecithin, polyoxyethylene lauryl ether, or the additive poly-L-lysine, but slightly affected by L-leucine, or colchicine. Results indicate that the transport of insulin across HIOEC monolayer is by paracellular pathway, and dependent of tight junctional characteristics.
The results showed that the permeated amount of insulin was significantly increased (p<0.01) in presence of chitosan, egg lecithin, and polyoxyethylene lauryl ether compared with that in presence of PBS at the same time point, respectively. Effects of the additives on the HIOEC monolayer are shown in Fig. 5B. The results showed that the permeated amount of insulin was significantly increased (p<0.01) in presence of poly-L-lysine but slightly affected in presence of L-leucine, and cholicine as compared with that in presence of PBS, respectively.

The effects of enhancers or additives on the apparent permeability coefficient (P_{app}) of insulin across HIOEC (human immortalized oral epithelial cells) monolayer are depicted in Table 5. Results showed the P_{app} values of insulin were significantly increased by the selected three enhancers, and an additive (poly-L-lysine), respectively. The rank order was as follows: chitosan (3.39±1.47×10^{-6} cm·s^{-1})>poly-L-lysine (2.70±0.57×10^{-6} cm·s^{-1})>egg lecithin (2.52±1.09×10^{-6} cm·s^{-1})>polyoxyethylene lauryl ether (2.47±1.17×10^{-6} cm·s^{-1}). The P_{app} values of insulin were not affected by the other two additives, colchicines, and L-leucine.

Fig. 6. (A) Plasma Glucose Level–Time Profiles after Insulin Solution (10 IU/kg) with an Enhancer Was Sublingually (sl) Administered to Normal Rats (A) Dose of 1 IU/kg insulin was subcutaneously (sc) injected into rats as a positive control. The enlarged figures are shown in (B) through (F), respectively. (B) Insulin (1 IU/kg, sc) versus insulin (10 IU/kg, sc); (C) insulin (10 IU/kg, sc) versus insulin (10 IU/kg with HP-β-CD (5%,w/v), or insulin (10 IU/kg, sc) with oleic acid (5%,w/v); (D) insulin (10 IU/kg, sl) versus insulin (10 IU/kg, sl) with egg lecithin (5%,w/v), or insulin (10 IU/kg, sl) with polyethylene polypropylene glycol (5%,w/v); (E) insulin (10 IU/kg, sl) versus insulin (10 IU/kg, sl) with polysorbate 80 (5%,w/v); (F) insulin (10 IU/kg, sl) versus insulin (10 IU/kg, sl) with chitosan (5%,w/v), or insulin (10 IU/kg, sl) with polyoxyethylene lauryl ether (5%,w/v); p<0.05, and ++p<0.01, versus insulin (10 IU/kg), respectively. The data are expressed as the mean±S.E.M. The results showed that the plasma glucose levels after administration of the insulin solution with an enhancer were very significantly lowered compared to those without an enhancer at the same time point, indicating that the enhancers could significantly increase the penetration of the insulin through sublingual mucosa in vivo.
In Vivo Hypoglycemic Activity

Plasma glucose level–time profiles after administrations of insulin with and without enhancers are shown in Fig. 6A, and the enlarged figures are depicted in B through F, respectively. The results showed that the plasma glucose levels were significantly reduced after sublingual administrations of insulin in presence of HP-β-CD, oleic acid, polysorbate 80, polyethylene–polypropylene glycol, egg lecithin, chitosan, or polyoxyethylene lauryl ether as compared with that in absence of an enhancer at the same time point, respectively. As a positive control, the plasma glucose levels were significantly decreased after subcutaneous injection of 1 IU/kg insulin. The difference value of the area above the plasma glucose curve (ΔAAC) were as follows: 3185.4±53.6 min·mmol/l for insulin with polyethylene–polypropylene glycol (sl)>1455.2±80.9 min·mmol/l for insulin with polyoxyethylene lauryl ether (sl, p<0.01)>1277.3±44.6 min·mmol/l for insulin with polyethylene–polypropylene glycol (sl, p<0.05)>1158.0±51.3 min·mmol/l for insulin with oleic acid (sl, p<0.05)>499.4±64.4 min·mmol/l for insulin with chitosan (sl, p<0.05)>423.5±62.8 min·mmol/l for insulin with HP-β-CD (sl, p>0.05)=423.5±62.8 min·mmol/l for insulin with polysorbate 80 (sl, p>0.05)>361.29±32.9 min·mmol/l for insulin with egg lecithin (sl, p<0.01)>1455.2±80.9 min·mmol/l for insulin with oleic acid, and both cyclodextrin derivatives possess the same lipophilic cavity. Egg lecithin may come from the mucosal lipids or directly inserted into the bilayer of mucosa, thus leading to the increased lipid fluidity of mucosa. As for chitosan, polyethylene–polypropylene glycol, polyoxyethylene lauryl ether, and oleic acid, they may directly insert into the lipid bilayer of mucosal cells because of the interaction of mucosal lipid with similar chemical group in the enhancer molecular structures, thus causing the reduced ΔAAC values due to disturbance of the well-organized bilayer, as described in the report.37) This postulation could be supported by the observation that polyethylene–polypropylene glycol can directly insert into lipid layer.38) In addition, as another support example, oleic acid that is called a mono-unsaturated fatty acid, was reported to be able to increase the permeation of lipophilic drugs through the skin and buccal mucosa by transcellular pathway.39) On the contrary, polysorbate 80 shows slight influence on the lipid bilayer, and the possible reason needs further investigation.

The proteins involved in the present conformation study may come from the bilayer and cell-to-cell junctions. Therefore, the changed structure of proteins could contribute to the increased fluidity of cellular membrane also, and the opened tight junction. As mentioned in introduction section, mucosal proteins are involved in the cell bilayer, adherent junction, and tight junction. Cell–cell adherent junctions are just one adhesion type including the desmosome and focal contact. Cell–cell adherent attach the actin filament system from one cell to that of a neighbouring cell.40) Tight junction is the cell–cell junction that seals adjacent epithelial cells together, preventing the passage of most dissolved molecules from one side of the epithelial sheet to the other. Therefore, proteins in tight junction may play a critical role for the transport of polypeptide across mucosa. There were no direct evidence to show how these enhancers interact with the protein secondary structure but our results demonstrate that all seven enhancers influence the secondary structure of mucosal proteins. The possible reasons for the changed secondary parameters, i.e. α-helix, β-sheet, β-turn, and random coil, could be derived from two aspects: one is the changed alignment of lipid molecules in the bilayer that cause steric alteration of the inserted mucosal protein, and another one is interaction.

### Table 6. The Difference Value of the Area above the Plasma Glucose–Time Curve (ΔAAC)

<table>
<thead>
<tr>
<th>Dose and administrations</th>
<th>ΔAAC (min. mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>101U/kg insulin, sl</td>
<td>361.29±32.9**</td>
</tr>
<tr>
<td>1U/kg insulin, sc</td>
<td>428.8±43.3**</td>
</tr>
<tr>
<td>101U/kg insulin with 5% HP-β-CD (w/v), sl</td>
<td>499.4±64.4**</td>
</tr>
<tr>
<td>101U/kg insulin with 5% oleic acid (w/v), sl</td>
<td>1277.3±44.6**</td>
</tr>
<tr>
<td>101U/kg insulin with 5 % egg lecithin (w/v), sl</td>
<td>3185.4±53.6</td>
</tr>
<tr>
<td>101U/kg insulin with 5% polyethylene–polypropylene glycol (w/v), sl</td>
<td>423.5±62.8**</td>
</tr>
<tr>
<td>101U/kg insulin with 5% polysorbate 80 (w/v), sl</td>
<td>1158.0±51.3**</td>
</tr>
<tr>
<td>101U/kg insulin with 5% chitosan (w/v), sl</td>
<td>1455.2±80.9**</td>
</tr>
<tr>
<td>101U/kg insulin with 5% polyoxyethylene lauryl ether (w/v), sl</td>
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</table>

All ΔAAC values were estimated versus the glucose level after insulin was sublingually administered to normal rats in absence of enhancer. Each value represents the mean±S.E.M. (n=5); **p<0.01, versus insulin with polyethylene–polypropylene glycol, Δp<0.05, versus insulin with polyoxyethylene lauryl ether; p<0.05, versus insulin with egg lecithin. Results indicate that seven enhancers significantly promote the hypoglycemic activity of insulin through sublingual delivery in normal rats as compared with insulin alone.

DISCUSSION

The reduced P and η values which indicate the increased fluidity of cellular membrane could be the result of mucosal disturbance derived from the changed order of lipid bilayer. The mechanisms of penetration enhancers influence the order or the structure may be through different approaches. Seven excipients were included in present investigation as the ‘penetration enhancers’, consisting of water soluble HP-β-CD, polyethylene–polypropylene glycol (poloxamer 188), polysorbate 80 (tween 80), polyoxyethylene lauryl ether (brij 35), chitosan (soluble in acid medium), amphipathic egg lecithin, and hydrophobic oleic acid. Our results show that six enhancers among the seven strongly influence the fluidity of sublingual mucosal lipid bilayer excluding polysorbate 80. The possible reasons may be as follows: HP-β-CD could extract the cholesterol from the membrane through forming stable complexes, which may cause the increase of fluidity and loosen the bilayer. The similar phenomenon was observed in DM-β-CD,35,36) and both cyclodextrin derivatives possess the same lipophilic cavity. Egg lecithin may come from the mucosal lipids or directly inserted into the bilayer of mucosa, thus leading to the increased lipid fluidity of mucosa. As for chitosan, polyethylene–polypropylene glycol, polyoxyethylene lauryl ether, and oleic acid, they may directly insert into the lipid bilayer of mucosal cells because of the interaction of mucosal lipid with similar chemical group in the enhancer molecular structures, thus causing the reduced P and η values due to disturbance of the well-organized bilayer, as described in the report.37) This postulation could be supported by the observation that polyethylene–polypropylene glycol can directly insert into lipid layer.38) In addition, as another support example, oleic acid that is called a mono-unsaturated fatty acid, was reported to be able to increase the permeation of lipophilic drugs through the skin and buccal mucosa by transcellular pathway.39) On the contrary, polysorbate 80 shows slight influence on the lipid bilayer, and the possible reason needs further investigation.

The proteins involved in the present conformation study may come from the bilayer and cell-to-cell junctions. Therefore, the changed structure of proteins could contribute to the increased fluidity of cellular membrane also, and the opened tight junction. As mentioned in introduction section, mucosal proteins are involved in the cell bilayer, adherent junction, and tight junction. Cell–cell adherent junctions are just one adhesion type including the desmosome and focal contact. Cell–cell adherent attach the actin filament system from one cell to that of a neighbouring cell.40) Tight junction is the cell–cell junction that seals adjacent epithelial cells together, preventing the passage of most dissolved molecules from one side of the epithelial sheet to the other. Therefore, proteins in tight junction may play a critical role for the transport of polypeptide across mucosa. There were no direct evidence to show how these enhancers interact with the protein secondary structure but our results demonstrate that all seven enhancers influence the secondary structure of mucosal proteins. The possible reasons for the changed secondary parameters, i.e. α-helix, β-sheet, β-turn, and random coil, could be derived from two aspects: one is the changed alignment of lipid molecules in the bilayer that cause steric alteration of the inserted mucosal protein, and another one is interaction.
between an enhancer and proteins. The steric alteration in cellular bilayer may possibly change the steric conformation of protein, and the direct interaction of an enhancer with the protein could affect the original secondary bonds that are for maintaining the steric conformation of protein, including hydrogen bond, hydrophobic bond, van der Waals forces, etc. thus affecting the protein secondary parameters. Our results demonstrate that the altered percentages for secondary parameters are varied when different enhancers are applied, indicating that the interaction forces and impacting degree may be varied with the structures of enhancers. The direct interaction between an enhancer and the proteins may cause cellular membrane alteration, and loosen the junctions, especially the tight junction. For increasing transport of polypeptide like insulin, the loosed tight junction may be a significant factor.

Insulin mainly exists in the hexamer conformation in the solution, and such a six-fold increase in molecular weight may restrict the penetration through the mucosa. Dissociation of insulin from hexamers to monomers could improve the mucosal penetration of insulin. Attenuation of the band in circular dichroism spectra is correlated with deaggregation of insulin, while intensification of the band is associated with enhanced association of monomers.

Our results indicated that three enhancers, *i.e.* egg lecithin, chitosan, and polyoxyethylene lauryl ether, could dissociate the hexamers of insulin to monomers but other four enhancers seemed not to affect the dissociation state of insulin. The possible reasons may be related to the surface-activity of the enhancers besides the reported impacting factors like concentration and pH value.

TEER values are reported to be related to the tight junction of monolayer. Our results showed that different insulin concentrations did not change the TEER values of HIOEC monolayer. Therefore, it could exclude the possibility that insulin itself open the tight junction of the monolayer. For the four selected enhancers, polyoxyethylene lauryl ether, and chitosan exhibited significant effect on tight junction. It has been reported that chitosan could loose the intercellular filaments, and chitosan-mediated tight junction disruption is caused by a translocation of tight junction proteins from the membrane to the cytoskeleton. As for the effect of polyoxyethylene lauryl ether, the reduced tight junction may also attribute to the same reasons. Although HP-β-CD and egg lecithin showed significant effects on the membrane lipid fluidity, these two enhancers exhibited little effects on the TEER values. This phenomenon is irregular, and may be postulated that the decreased TEER values could not fully correlate with the increased membrane lipid fluidity, *viz.*, in some cases, although the well-organized membrane is disturbed either from bilayer or cell-to-cell junction, the TEER value may not be significantly changed. For an example, egg lecithin may interfere with the TEER value.

Our investigations further excluded the influences of experimental factors on the insulin transport across the HIOEC monolayer, namely, the transport directions, experimental temperatures, and insulin concentrations did not affect the *P<sub>app</sub>* value. But the selected three enhancers, chitosan, egg lecithin, and polyoxyethylene lauryl ether, significantly promoted the permeated amount of insulin and the *P<sub>app</sub>* value, indicating that the effect was from the enhancer. Our result is coincidence with the previous report that egg lecithin was able to enhance increases the iontophoretic transdermal mannitol flux about three-fold. The possible mechanisms may be due to triple aspects: increased membrane lipid fluidity, dissociation of insulin hexamers, and directly opened tight junction. Because the increased membrane lipid fluidity also results in the opened tight junction besides of the loosed lipid membrane, the most significant factors for increasing the transport of insulin could be from both the dissociation and the opened tight junction.

For characterizing the transport pathway of insulin, three additives, *i.e.*, L-leucine, cholicchicine, and poly-L-lysine, were coadministered with insulin, respectively. L-Leucine was reported to be actively transported across mucosa by consuming energy of ATP. Our results indicate that there is no competition phenomenon when insulin was coadministered with L-leucine, suggesting that insulin across HIOEC monolayer may not be through the active transport. Cholicchicine, a cell microtubule antagonist, was reported to disrupt the microtubule integrity in the cultured thyroid cell monolayer. It was included in the present study with the aim to understand whether cholicchicine also disrupt the tight junction of HIOEC monolayer. The permeated amount of insulin and the *P<sub>app</sub>* value were not significantly enhanced by the cholicchines, indicating that cholicchines may not affect the tight junction of HIOEC monolayer. This may be due to the different monolayer used in the present study. Poly-L-lysine, a cationic amino acid, could conjugate with the anionic ions on the surface of mucosa and alter the tight junction of cells leading to an increase of permeated drug amount or an enhanced *P<sub>app</sub>* value. Our result was consistent with these reports, indicating that insulin permeability is also associated with the tight junctional of monolayer.

The in vivo data showed that the hypoglycemic activity of insulin coadministered with any one of seven enhancers was significantly increased as compared with that of blank control (administered as insulin PBS solution alone). In view of the in vitro results, the promoted activity in vivo may be associated with the three aspects: increased lipid fluidity derived from lipid membrane disturbance or from conformation change of proteins inserted in the bilayer and the cell-to-cell junctions, dissociation of insulin hexamers to monomers, and directly opened tight junction. For the transport of polypeptide insulin, it could be postulated that the dissociation of insulin aggregates to monomers, and the opened tight junction would correlate most with the enhanced permeability or the increased hypoglycemic activity.

In conclusion, HP-β-CD, chitosan, polylethylene–polypropylene glycol, polysorbate 80, polyoxyethylene lauryl ether, egg lecithin, and oleic acid could significantly increase the mucosal lipid fluidity, affect the conformation of mucosal protein, and increase the transport amount of insulin or the apparent permeability (*P<sub>app</sub>* value), thus leading to the significantly increased hypoglycemic activity of insulin through sublingual delivery in normal rats. The mechanisms of the enhancing effects may be due to either one or multiple factors: increasing the mucosal lipid fluidity, direct loosening the tight junction of sublingual epithelia, and dissociating the hexamers of insulin to monomers. Among these, the opened tight junction may correlate most with the enhancing effect in the membrane permeability. Because the aggregates of insulin exist, the dissociation of the aggregates by an enhancer
would benefit the permeability.

REFERENCES