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Research Paper

Region-Dependent Role of the Mucous/Glycocalyx Layers in Insulin Permeation Across Rat Small Intestinal Membrane

Yoshinobu Aoki,[1,2] Mariko Morishita,[1,5] Kazunori Asai,[3] Bunshiro Akikusa,[4] Shuji Hosoda,[4] and Kozo Takayama[1]

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The regional difference in the contribution of the mucous/glycocalyx layers in rat small intestine, as a diffusional or enzymatic barrier, to the absorption of insulin was investigated by *in vitro* studies. The mucous/glycocalyx layers from the duodenum, the jejunum, and the ileum in rat were successfully removed without damaging membrane integrity, by exposing them to a hyaluronidase solution *in situ*. In an *in vitro* transport experiment, the apparent permeability coefficient (P_{app}) of insulin for the hyaluronidase-pretreated group was significantly increased compared to the PBS-pretreated (control) group in all small intestinal regions, and the P_{app} of insulin in both PBS- and hyaluronidase-pretreated groups increased in the following order: duodenum < jejunum < ileum. On the other hand, irrespective of small intestinal regions, the P_{app} of FD-4 and of antipyrine, respectively the passive para- and transcellular permeation marker, exhibited no significant differences between PBS- and hyaluronidase-pretreated group. In addition, a significant amount of insulin was degraded in the mucous/glycocalyx layers compartment removed by hyaluronidase pretreatment, and the degradation activity in the mucous/glycocalyx layers showed regional differences in the following order: duodenum > jejunum > ileum. These findings suggest that, irrespective of small intestinal regions, the mucous/glycocalyx layers contributed to insulin permeation predominantly as an enzymatic barrier, and not as a diffusional barrier. Furthermore, the variation of the enzymatic activities in the mucous/glycocalyx layers and in the brush-border membrane would be one factor that accounts for the regional differences in the transport of insulin.

KEY WORDS: glycocalyx; hyaluronidase; insulin; intestinal metabolism; macromolecular drug delivery; mucous; small intestine.

INTRODUCTION

In recent years, numerous candidates for novel therapeutic peptides have been created against a backdrop of rapid progress in the field of bio- as well as gene technology. Although the peroral administration of peptide drugs would be the most desirable alternative route to parenteral administration, the oral bioavailability of these drugs is generally poor because multiple physical and enzymatic barriers are encountered within the gastrointestinal tract. Thus, to develop therapeutically effective oral dosage forms for peptide drugs, as a first step, it is essential to understand which barrier(s) have important roles in peptide absorption and how they limit absorption from the intestine, which involves proteolytic degradation mechanisms and kinetics by secreted and membrane-bound proteases, as well as segmental differences in degradation rate and intestinal permeability.

The glycocalyx layer lines the intestinal epithelium and is covered by a loosely adherent mucous layer, with thicknesses of 0.1–0.5 and 5–10 μm, respectively (1,2). The mucous layer consists of glycoproteins, enzymes, electrolytes, and water, whereas the glycocalyx is primarily composed of glycoproteins, proteoglycans, and glycolipids including hyaluronan (3–11). Together, they are believed to protect the apical cell surface against microbial pathogens and foreign materials partially by virtue of the electrical repulsion for negatively charged sugar moieties (8,12). Also, these layers have been considered a diffusional barrier to the transport of peptides because the diffusing peptide molecules may interact with the components of mucous, due to the intrinsic physicochemical characteristics of most peptides, such as a higher molecular weight and hydrophilicity (13,14). On the other hand, it has been reported that these layers enmesh some endogenous constituents, including pancreatic enzymes capable of degrading certain peptides (5). Notably, the mucous/glycocalyx layers seem to contribute to the absorption of certain peptides as an enzymatic barrier and/or diffusional barrier.

[1] Department of Pharmaceutics, Hoshi University, Ebara 2-4-41, Shinagawa, Tokyo 142-8501, Japan.
[2] Research Center, Kyorin Pharmaceutical Co., Ltd., Nogi 1848, Nogi-machi, Shimotsuga-gun, Tochigi 329-0114, Japan.
[3] Department of Pharmacy, Matsudo City Hospital, Kamihongou 4005, Matsudo, Chiba 271-8511, Japan.
[4] Department of Pathology, Matsudo City Hospital, Kamihongou 4005, Matsudo, Chiba 271-8511, Japan.
[5] To whom correspondence should be addressed. (e-mail: morisita@hoshi.ac.jp)

In a previous study, we demonstrated that rat ileal mucous/glycocalyx layers could be removed by hyaluronidase, an enzyme capable of reducing the glycocalyx component, without causing detectable cellular damage. Also, we demonstrated in the rat ileum, using an *in vitro* and an *in situ* system, that the mucous/glycocalyx layers played a significant role in the absorption of insulin, which was chosen as a model peptide, (15,16). On the other hand, there were some reports that the apparent permeability of insulin differs among various intestinal regions (17,18). These results imply that the impedance of the mucous/glycocalyx layers to insulin absorption would be varied among different small intestinal regions. In addition, collecting information about the regional differences would be helpful in designing reasonable strategies to improve peroral insulin absorption. Therefore, the aim of this study was to extend the application of hyaluronidase treatment to other parts of the small intestine to remove the mucous/glycocalyx layers without causing detectable damage to the membrane integrity, and also, to investigate the regional differences in the contribution of the mucous/glycocalyx layers, as a diffusional and/or enzymatic barrier, to insulin absorption by *in vitro* permeation experiments.

MATERIALS AND METHODS

Materials

Crystalline human recombinant insulin (USP; 28.6 IU/mg), fluorescein isothiocyanate (FITC)-labeled dextran with a weight-average molecular weight (M_W) of 4.4 kDa (FD-4), lyophilized hyaluronidase (EC 3.2.1.35; Type IV-S from bovine testes; M_W = 56 kDa, 1,320 U/mg solid), and sodium taurodeoxycholate were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Antipyrine was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of analytical grade and used as received from the suppliers.

In Situ Hyaluronidase Pretreatment

This research was performed at Hoshi University and complied with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals. Male Wistar rats weighing 180–220 g were purchased from Sankyo Lab Service (Tokyo, Japan). The animals were housed in rooms maintained at 23 ± 1°C and 55 ± 5% relative humidity, and allowed free access to water and food during acclimatization. All animals were fasted for 24 h prior to experiments. Following anesthetization with an intraperitoneal injection of sodium pentobarbital (50 mg/kg; Dainippon Pharmaceutical, Osaka, Japan), rats were restrained in a supine position on a thermostatically controlled board at 37°C. The hyaluronidase pretreatment conditions, e.g., the concentration of hyaluronidase and exposure time, were selected based on the conditions optimized in a previous study using rat ileum (16). Briefly, the small intestine was exposed following a midline incision carefully made in the abdomen, and loops of the duodenum, the jejunum, and the ileum were cannulated at both ends using polypropylene tubing (4 mm OD, 2 mm ID; Saint-Gobain Norton, Nagano, Japan). The duodenal loop was made at the first portion of the intestine closest to the stomach (ca. 6 cm in length). The next portion, 5 cm away from the ligament of Treitz, was utilized as the jejunal loop (ca. 10 cm in length). The ileal loop was made at the end of the small intestine, just proximal to the ileo–cecal junction (ca. 10 cm in length). Subsequently, these loops were securely ligated to prevent fluid loss. The loops were gently rinsed with 20 mL of phosphate-buffered saline (PBS; pH 7.4) at 37°C to remove luminal enzymes, then exposed to 1.0 mL (37°C) of PBS (control) or hyaluronidase in PBS at 192,000 U/mL for 30 min, and tightly closed at both ends. Next, the loops were carefully reinserted into the abdominal cavity, and the abdominal wall was sutured to prevent heat loss. At the end of the exposure, the loops were gently rinsed with 20 mL of PBS at 37°C. Before and after the pretreatment with hyaluronidase, the rinse solutions were collected to determine the peptidolytic activity as described below. The rinse solutions were centrifuged for 10 min (4°C, 2,700×*g*) and the supernatant was removed for use in the peptidolytic degradation experiment. In the case of the duodenal loop, the bile duct was ligated before the pretreatment.

Transmission Electron Microscopy

The pretreated intestinal segments were removed from the body and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). A secondary fixation employed 1.0% osmium tetroxide in the same buffer for 1 h, followed by dehydration and embedding in Epon 812 (NISSIN-EM, Tokyo, Japan). Thin cross-sectional samples were prepared by an ultramicrotome (ULTRACUT N, Reichert-Nissei, Tokyo, Japan). Samples were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy (TEM) (H-7000, Hitachi Ltd., Tokyo, Japan) to evaluate the diminishment of the mucous/glycocalyx layers.

Histological and Biochemical Examination of the Intestinal Membranes Following Hyaluronidase Pretreatment

The intestinal segment of each region was pretreated with PBS (negative control) or hyaluronidase in PBS as described above. Positive controls were pretreated with 1.0% (w/v) sodium taurodeoxycholate in PBS because of its known induction of mucosal damage (19).

Light Microscopy

The intestinal segments were removed following the pretreatment and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Subsequently, thin cross-sectional samples were prepared via the microtome, followed by staining for light microscopic observation. Samples were stained with hematoxylin–eosin stain and with alcian-blue stain at pH 2.5 (Kanto Chemical, Tokyo, Japan) to histologically assess tissue damages and to assess the diminishment of the mucous/glycocalyx layers, respectively (20).

Lactate Dehydrogenase Leakage

Following the pretreatment, the rinse solutions were collected to determine the leakage of lactate dehydrogenase

(LDH), an intracellular enzyme often used to evaluate intracellular integrity (21). Lactate dehydrogenase was quantified using the LDH Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan) following in-house validation that had ensured a lack of assay interference by the presence of hyaluronidase in the sample solutions.

In Vitro Transport Experiments

In vitro transport experiments were performed with an Ussing chamber using the intestinal membranes pretreated with either PBS (control) or hyaluronidase according to a previously described method (16). Briefly, following pretreatment as described above, the segments were opened along the mesenteric border and carefully washed with ice-cold Krebs–Ringer's (bicarbonate buffered) solution (pH 7.4) containing 0.001% methylcellulose to prevent the adsorption of insulin by the surface of the chamber and container. Krebs–Ringer's solution (in mM) was composed of 108.0 NaCl, 11.5 D-glucose, 15.0 $NaHCO_3$, 4.7 KCl, 1.8 NaH_2PO_4, 0.4 KH_2PO_4, 1.2 $MgSO_4$, 1.25 $CaCl_2$, 4.9 Na glutamate, 5.4 Na_2 fumarate, and 4.9 Na pyruvate. Subsequently, their muscle layer was stripped away, and the (flat sheet) membranes were mounted in an Ussing chamber exposing a surface area of 1.0 cm^2 (CEZ-9100; Nihon-Kohden Tokyo, Tokyo, Japan). Both the mucosal side (donor side) and serosal side (receiver side) were filled with 5.0 mL of Krebs–Ringer's solution at 37°C and continuously bubbled with a gas mixture containing 95% O_2 and 5% CO_2. Then the intestinal membranes were allowed to equilibrate for 20 min. FD-4, a stable hydrophilic compound with a comparable molecular weight to insulin, and antipyrine, a lipophilic compound, were selected as passive para- and transcellular transport markers, respectively. It was suggested in several studies that some of the commercially available FITC-labeled dextrans (FDs) supplied by Sigma-Aldrich contain free FITC. In fact, in some cases, further purification is required for the study (22). In this study, however, the high purity of FD-4 was confirmed by chromatographic technique (data not shown), and these results are consistent with other reports (22,23). Therefore, the influence of FD-4 impurity on the permeation coefficients, if any, can be assumed as negligible. Thus, FD-4 was not further purified in this study.

Various drug solutions were prepared with Krebs–Ringer's solution. After an equilibration period, the permeation experiment was started by replacing 1 mL of the solution in the mucosal side with an equal volume of drug solution, and the final concentration in the donor side was adjusted to 200 or 1,000 μM for insulin, 10 mg/mL for FD-4, and 4 mg/mL for antipyrine. The preparation of the insulin solution was as follows: insulin was initially dissolved in 0.1 M HCl, then Krebs–Ringer's solution was added to yield a predetermined concentration; the solution's pH was simultaneously adjusted to 7.4, using 0.1 M NaOH. At a predetermined time, samples (100 μL) were taken from the serosal side and immediately replaced with an equal volume of Krebs–Ringer's solution.

To assess tissue viability throughout the experiment, the spontaneous transmucosal potential difference (PD) and the short circuit current (I_{sc}) were simultaneously monitored every 10 min during the experiment. The values for R_m were calculated by PD/I_{sc}, based on Ohm's law. These were corrected by eliminating the offset voltage between the electrodes and series fluid resistance, which was determined prior to each experiment using the same bathing solutions, yet in the absence of the membranes mounted in the chamber. The values for R_m of used intestinal membrane in steady state were in the range of 50–90, 40–70, and 30–50 Ω cm^2 in the duodenum, the jejunum, and the ileum, respectively. The values for R_m were greater than 80% of the start value at the end of each test period, and the course of R_m during the test period showed no difference between PBS- and hyaluronidase-treated groups, denoting that the viability of the intestinal membrane was maintained.

Insulin concentration was measured via an immunochemiluminometric assay using a microplate luminometer (Mithras LB940; Beltold Japan, Osaka, Japan). FD-4 concentration was determined by using a microplate luminometer at excitation and emission wavelengths of 485 and 535 nm, respectively. Antipyrine concentration was determined by HPLC. The HPLC system consisted of a Shimadzu SCL-10A system controller, an SIL-10A autoinjector, an LC-10AS liquid chromatograph, an SPD-6A UV spectrophotometric detector, and a C-R6A chromatopac. The mobile phase was a mixture of 6.7 mM phosphate buffer, pH 7.2, and acetonitrile (100:18). The sample (20 μL) was injected onto an Inertsil C-8 column (250 × 4.6 mm). The flow rate was 2 mL/min and the ultraviolet/visible detector was set at 254 nm.

The steady-state flux (J_{ss}) was calculated from the linear portion of the cumulative flux-*vs.*-time curve. The apparent permeability coefficient (P_{app}) of each compound was calculated as shown below:

$$P_{app} = J_{ss}/C$$

where C is the initial donor concentration.

The apparent permeation resistance in the mucous/glycocalyx layers ($R_{m/g}$) and the mucosal membrane (R_{mem}) in total apparent permeation resistance (R_{total}) are expressed as follows:

$$R_{total} = R_{m/g} + R_{mem}$$

where R_{total} and R_{mem} are derived from the inverse of P_{app} for PBS-pretreated (control) group ($P_{app_{(PBS)}}$) and hyaluronidase-pretreated group ($P_{app_{(Hyl)}}$), respectively.

Degradation of Insulin in Rinse Solutions

The degradation of insulin in the rinse solutions, collected before and after the pretreatment as described above, was studied by incubating 5 mL of the rinse solution with 100 μL of the insulin solution (600 μM) at 37°C according to a previously described method (16). The insulin solution (600 μM) was prepared as described for the *in vitro* transport experiments. At predetermined times, up to 120 min, 100 μL aliquots of solution were withdrawn from the incubation mixture and immediately added to 500 μL of a 1% TFA solution to terminate the reaction. The samples were subsequently stored in a freezer at −80°C until HPLC analysis. HPLC analysis was performed with a Waters Alliances HPLC system (Nihon Waters, Tokyo, Japan). The

gradient system consisted of mobile phase A; water containing 0.1% trifluoroacetic acid, and mobile phase B; 100% acetonitrile. The system was programmed so that the proportion of mobile phase B increased from 22 to 40% within 32 min. The sample (20 μL) was injected onto an Inertsil C8 column (250 × 4.6 mm) connected to a C8 precolumn. The gradient mobile phase was run at a flow rate of 1 mL/min and the ultraviolet/visible detector was set at 210 nm.

Statistical Analysis

Each value was expressed as the mean ± standard error (SE). Statistical significance was assayed by a Student's *t* test; *p* values below 0.05 were considered significant.

RESULTS

Mucous/Glycocalyx Layers Following Hyaluronidase Pretreatment

Figures 1 and 2 show light micrographs (alcian blue stain) and electron micrographs of the PBS-treated and the hyaluronidase-treated intestinal mucosal membranes in the duodenum, the jejunum, and the ileum. From light microscopic observations (alcian blue stain), intense staining along the apical side of the epithelial membranes was clearly observed for the PBS-treated (control) group irrespective of intestinal regions, indicating the existence of the glycocalyx. In contrast, such a layer was hardly observed for the hyaluronidase-treated group irrespective of intestinal regions; most of the mucous layer was removed via chemical fixations and dehydration of the intestinal segment samples, and thus the mucous layer was unobserved in any specimens. From the electron microscopic observations, approximately 70- to 100-nm thickness of the glycocalyx layer was clearly observed as electron-dense layers, enveloping the microvillus consistently for PBS-treated (control) group irrespective of intestinal regions. In contrast, hyaluronidase pretreatment was found to remarkably diminish such a layer, resulting in a near-naked microvillus.

Histological and Biochemical Examinations of Small Intestinal Membranes Following Hyaluronidase Pretreatment

Figure 3 shows light micrographs (hematoxylin–eosin stain) of the PBS-treated and the hyaluronidase-treated intestinal mucosal membranes in the duodenum, the jejunum, and the ileum. For each small intestinal region, no apparent histological damage was found in the hyaluronidase-treated mucosal membranes and cells as well as PBS-treated (control) counterparts.

Table I shows the LDH leakage for PBS and hyaluronidase pretreatment irrespective of small intestinal regions. Following hyaluronidase pretreatment, LDH leakage into the mucosal lumen was negligible, which was similar to the amount of leakage observed in the PBS-treated group. In contrast, LDH leakage was dramatically increased with 1.0% (w/v) sodium taurodeoxycholate.

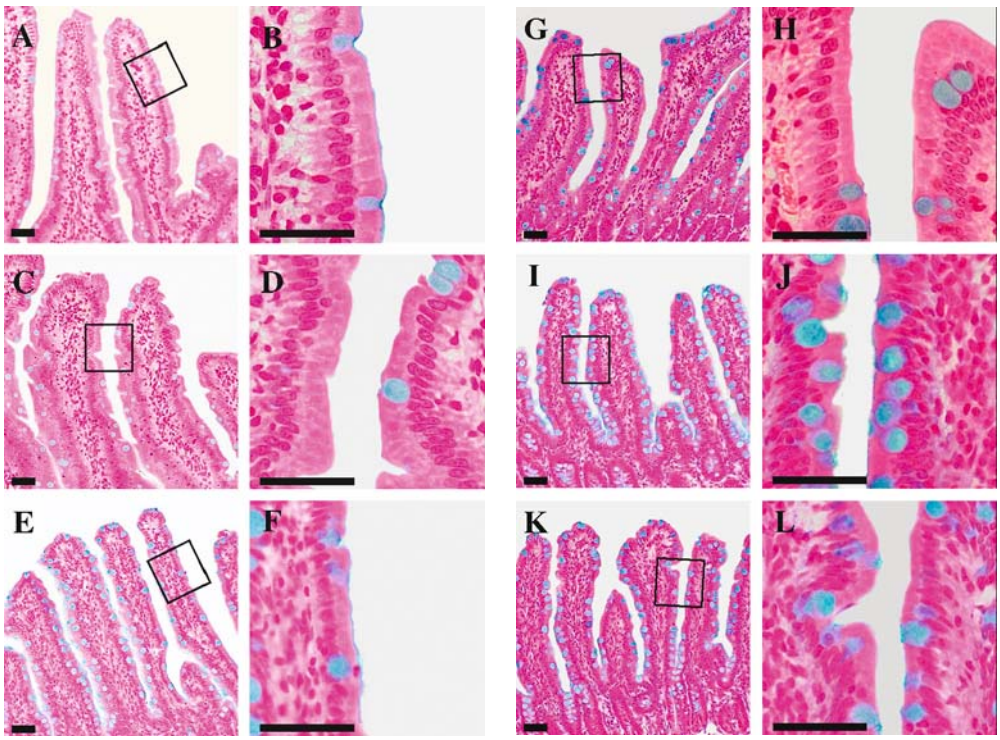

Fig. 1. Light micrographs of small intestinal mucosa pretreated with PBS (A, B, E, F, I, and J) and hyaluronidase at 192,000 U/mL (C, D, G, H, K, and L): duodenum (A and C, ×10; B and D, × 40), jejunum (E and G, ×10; F and H, ×40), ileum (I and K ×10; J and L, ×40). The scale bar (thick bar) was 40 μm. Tissues were stained with alcian blue (pH 2.5) following fixation using paraformaldehyde (2.0%).

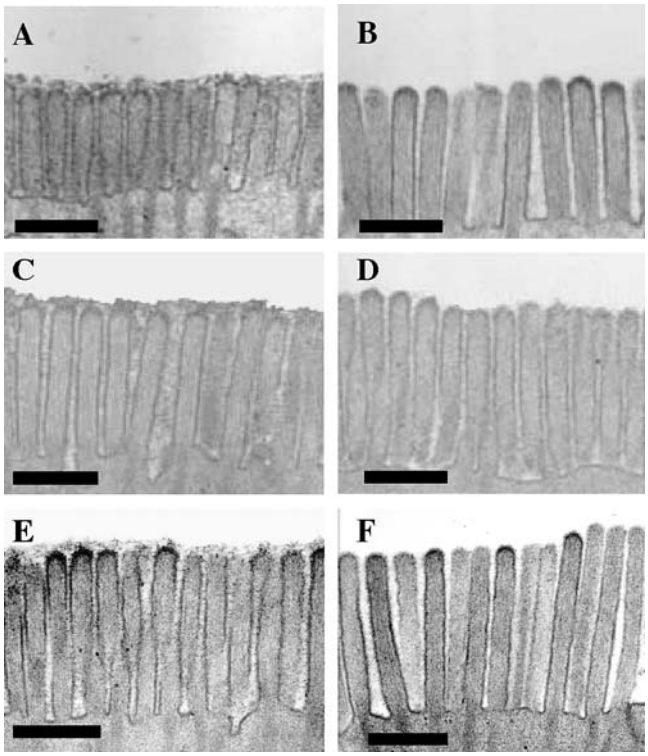

Fig. 2. Electron micrographs of small intestinal mucosal membranes pretreated with PBS (A, C, and E) and hyaluronidase at 192,000 U/mL (B, D, and F): duodenum (A and B), jejunum (C and D), and ileum (E and F). The scale bar (thick bar) was 0.5 μm. Tissues were stained with uranyl acetate and lead citrate following primary and secondary fixations using glutaraldehyde (2.5%), and osmium tetroxide (1.0%).

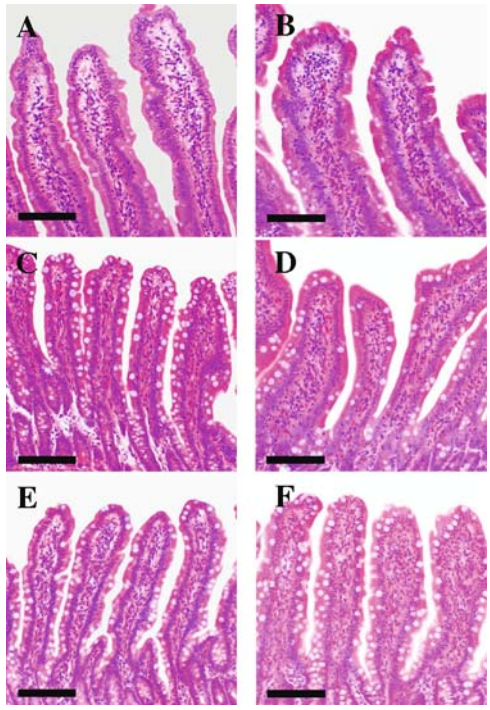

Fig. 3. Light micrographs of small intestinal mucosa pretreated with PBS (A, C, and E) and hyaluronidase at 192,000 U/mL (B, D, and F): duodenum (A and B), jejunum (C and D), and ileum (E and F). The scale bar (thick bar) was 100 μm. Tissues were stained with hematoxylin and eosin following fixation using paraformaldehyde (2.0%).

In Vitro Transport Experiment

Figure 4, 5, and Table II show the transport profiles and the apparent permeability coefficient (P_{app}) of insulin, FD-4, and antipyrine across the various intestinal membranes pretreated with PBS and hyaluronidase. The P_{app} of insulin for the hyaluronidase-treated group was significantly increased compared to the PBS-treated (control) group in all regions at a donor side concentration of 1,000 μM. Note that at a donor side concentration of 200 μM, only a small amount of insulin appeared on the receiver side. Consequently, the steady-state flux (J_{ss}) could not be calculated; thus only the P_{app} at a donor side concentration of 1,000 μM are shown in Table II. The P_{app} of insulin in both PBS-pretreated (control) and hyaluronidase groups increased in the following order: duodenum < jejunum < ileum. Values ranged from 0.05×10^{-8} to 0.47×10^{-8} cm s^{-1} for the PBS-pretreated group and from 0.14×10^{-8} to 0.95×10^{-8} cm s^{-1} for the hyaluronidase-pretreated group. On the other hand, irrespective of small intestinal regions, the P_{app} of FD-4 and antipyrine exhibited no significant differences between PBS-pretreated (control) and hyaluronidase-pretreated group. The P_{app} of FD-4 in both PBS- and hyaluronidase-pretreated groups slightly increased in the following order: duodenum ≤ jejunum ≤ ileum; however, the values were not significantly different. The P_{app} order of antipyrine in both PBS-pretreated (control) and hyaluronidase-pretreated groups was duodenum = jejunum < ileum, of which the values in the ileum were significantly different than those in the duodenum and the jejunum.

The percentage ratio of $R_{m/g}$ to R_{total} is regarded as an index of the influence of the mucous/glycocalyx layers to the whole process of drug permeation across the intestinal mucosa. In particular, the percentage ratio of $R_{m/g}$ to R_{total} for insulin were prominent: 62.2, 48.9, and 50.1% for the duodenum, the jejunum, and the ileum, respectively. On the other hand, the percentage ratio of $R_{m/g}$ to R_{total} for FD-4 and antipyrine were modest: 4.4, −0.3, and −2.3% (FD-4); 11.5, 7.5, and 2.6% (antipyrine) for the duodenum, the jejunum and the ileum, respectively.

Table I. Lactate Dehydrogenase (LDH) Leakage Following PBS (Control), Hyaluronidase and 1.0 % (w/v) Sodium Taurodeoxycholate Pretreatment

	LDH leakage [U]		
	Control	Hyaluronidase	Sodium taurodeoxycholate
Duodenum	2.6 ± 0.8	2.2 ± 0.8	19.7 ± 5.7*
Jejunum	2.5 ± 0.6	2.3 ± 0.1	30.3 ± 1.9*
Ileum[a]	1.3 ± 0.3	1.0 ± 0.2	12.3 ± 1.6*

[a] These values are from our previous report (16). Each value represents the mean with standard error of group of three to four experiments.
*$p < 0.05$ against control.

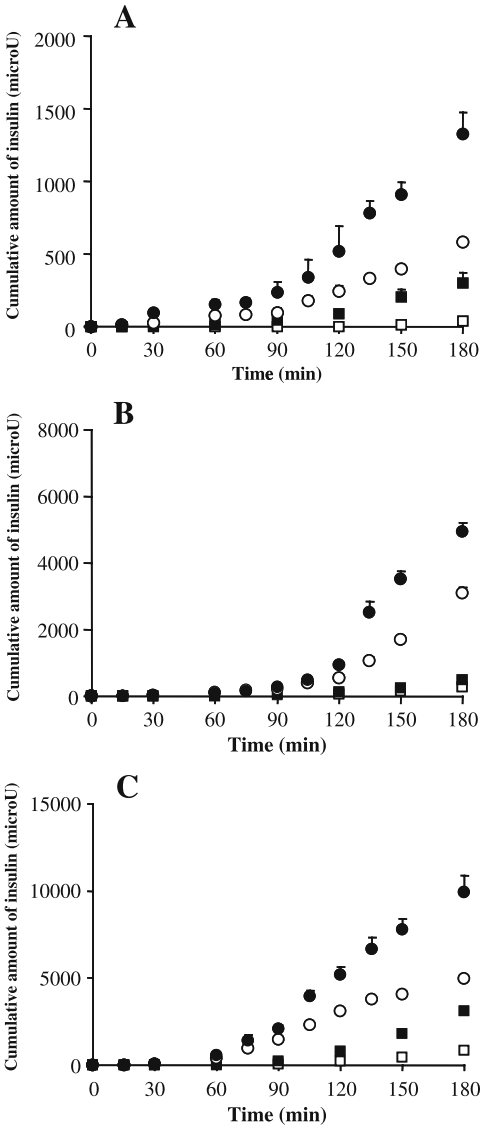

Fig. 4. Time course of insulin transport across rat small intestinal segments pretreated with PBS and hyaluronidase at 192,000 U/mL: (A) duodenum, (B) jejunum, (C) ileum. PBS (open symbol), hyaluronidase (closed symbol); donor concentration of insulin at 200 μM (□, ■) and at 1,000 μM (○, ●). Data represent the mean with standard error of group of four to seven experiments.

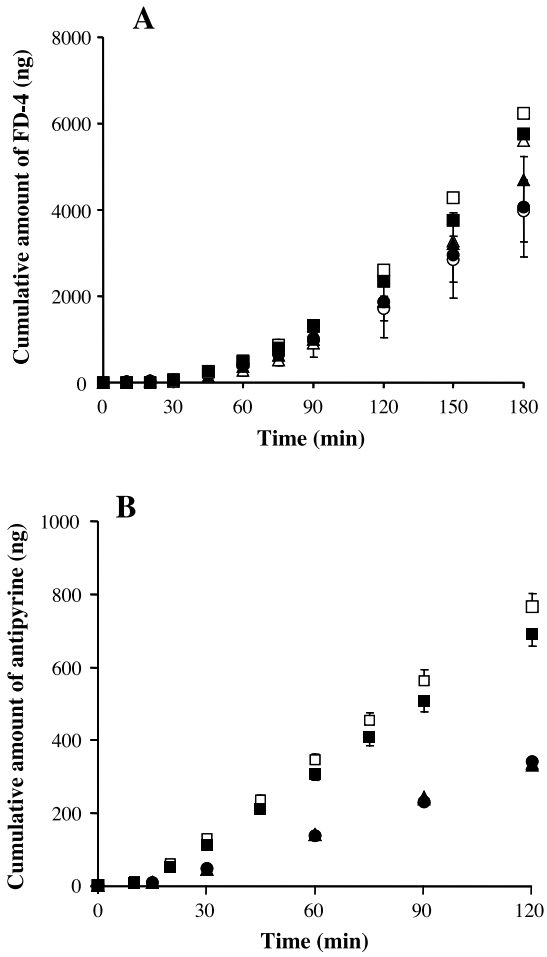

Fig. 5. Time course of (A) FD-4 and (B) antipyrine transport across rat small intestinal segments pretreated with PBS and hyaluronidase at 192,000 U/mL. PBS (open symbol), hyaluronidase (closed symbol); (○, ●) duodenum, (△, ▲) jejunum, (□, ■) ileum. Data represent the mean with standard error of group of four to seven experiments.

Stability of Insulin in the Rinse Solution

Figure 6 and Table III show the profiles and half-lives of the degradation of insulin in the rinse solution collected after pretreatment. For all regions, a substantial amount of insulin was degraded in the rinse solution collected after the pretreatment with hyaluronidase. The half-life calculated from the apparent first-order rate constant for the degradation of insulin in the rinse solution collected after the hyaluronidase pretreatment was significantly shorter than that in the solution collected after PBS pretreatment (54 *vs.* 428, 61 *vs.* 400, and 111 *vs.* 386 min for hyaluronidase- *vs.* PBS-treated groups in the duodenum, the jejunum, and the ileum, respectively; $p < 0.05$). The half-lives in the duodenum and the jejunum were almost the same, but the rinse solution for the duodenum was more diluted than for the jejunum

Table II. Apparent Permeability Coefficient of Insulin, FD-4 and Antipyrine during Transport Across the Various Small Intestinal Membranes with or without Hyaluronidase Pretreatment

		Apparent permeability, P_{app} (10^{-8} cm/s)		
		Pretreatment		
Solute	Region	PBS	Hyaluronidase	Ratio
Insulin	Duodenum	0.054 ± 0.007	0.143 ± 0.016*	2.7
	Jejunum	0.334 ± 0.022	0.654 ± 0.032*	2.0
	Ileum	0.474 ± 0.020	0.950 ± 0.040*	2.0
FD-4	Duodenum	43.08 ± 3.54	48.38 ± 13.88	1.1
	Jejunum	56.11 ± 5.36	55.96 ± 3.23	1.0
	Ileum[a]	61.44 ± 3.54[a]	60.04 ± 6.05[a]	1.0
Antipyrine	Duodenum	125.0 ± 6.2	140.7 ± 4.9	1.1
	Jejunum	143.3 ± 7.9	133.3 ± 7.0	0.9
	Ileum[a]	264.8 ± 27.1[a]	272.0 ± 15.8[a]	1.0

[a] These values are from our previous report (16). Each value represents the mean with standard error of group of four to seven experiments.
*$p < 0.05$ against control.

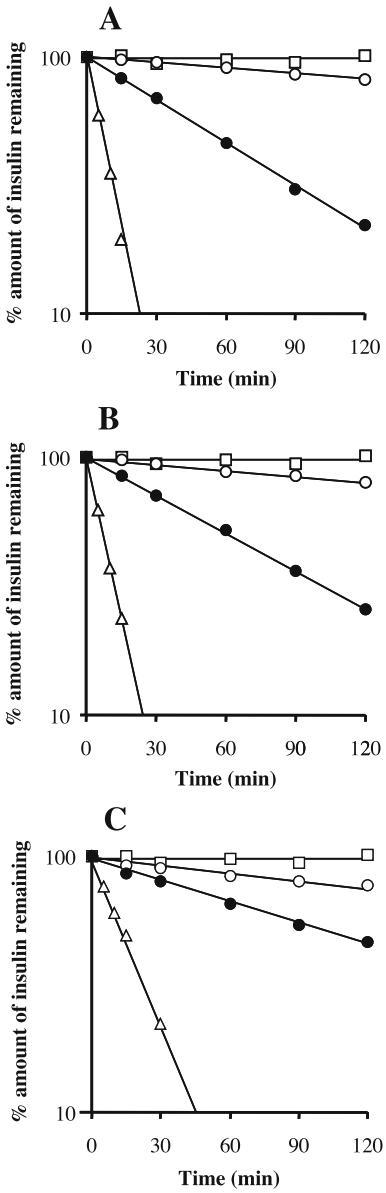

Fig. 6. Degradation profile of insulin as a function of time in the rinsed-out solutions: (A) duodenum, (B) jejunum, (C) ileum. (□) PBS solution (control), (○) PBS pretreatment, (●) hyaluronidase pretreatment, and (△) before pretreatment (ten times dilution). Data represent the mean with standard error of group of three to four experiments.

because the volume of rinse solution per segment length for the duodenum (20 mL/6 cm) was higher than that for the jejunum (20 mL/10 cm). Thus, the enzymatic degradation activity in the hyaluronidase group for the duodenum was actually higher than that for the jejunum. Consequently, the enzymatic degradation activity in the hyaluronidase groups increased in the following order: duodenum > jejunum > ileum. Meanwhile, in the rinse solution collected before the pretreatment, i.e., the luminal fluid, insulin was quickly degraded, although the solution was diluted tenfold (6.5 ± 0.4, 7.2 ± 0.2, and 14.0 ± 0.1 min in the duodenum, the jejunum, and the ileum, respectively). This implies that extensive proteolytic degradation occurred in the intestinal tract in a very short time.

Table III. Half-Lives for Degradation of Insulin in Rinse Solutions Collected after the Pretreatment

Intestinal region	Pretreatment	Half-lives (min)
Duodenum	PBS	427.7 ± 72.8
	Hyaluronidase	54.2 ± 2.2*
Jejunum	PBS	399.6 ± 78.4
	Hyaluronidase	61.4 ± 3.5*
Ileum	PBS	385.8 ± 92.0
	Hyaluronidase	110.5 ± 5.1*

Each value represents the mean with standard error of group of four experiments.
*$p < 0.05$ against control (PBS pretreatment).

DISCUSSION

Our previous study demonstrated that hyaluronidase pretreatment successfully removed the mucous/glycocalyx layers of the rat ileum. In this study, we extended the application of hyaluronidase pretreatment to other parts of the small intestine and demonstrated that hyaluronidase pretreatment immutably removed the mucous/glycocalyx layers of each small intestinal segment without damaging membrane integrity as shown by microscopic observations and LDH leakage values (Figs. 1–3, Table I). Therefore, irrespective of small intestinal regions, our hyaluronidase pretreatment technique would allow for precise investigation into the contribution of the mucous/glycocalyx layers to the transport of certain drugs across the small intestinal mucosa.

In this study, we observed an increase in the P_{app} of insulin by removal of the mucous/glycocalyx layers, irrespective of small intestinal regions. These results concur with our previous study using only the ileal segment. P_{app} in the PBS-pretreated groups were less than the reported P_{app} of insulin from rat small intestine for the duodenum, the jejunum, and the ileum ($0.78 \pm 0.54 \times 10^{-7}$, $4.97 \pm 1.51 \times 10^{-7}$, and $6.82 \pm 1.87 \times 10^{-7}$ cm s^{-1}; $0.485 \pm 0.099 \times 10^{-6}$, $1.227 \pm 0.173 \times 10^{-6}$, and $1.050 \pm 0.206 \times 10^{-6}$ cm s^{-1}) (17,18). Variation in P_{app} could be explained by differences in apparatus, tissue preparation, concentrations of insulin in donor side studied, analytical method employed, and the duration of experiments.

Generally, for hydrophilic macromolecules, such as insulin and FD-4, the most likely route of transport from the mucosal side to the serosal side is the paracellular pathway due to their macromolecular weight. In this putative route, permeation resistances of the mucous/glycocalyx layers and the intrinsic intestinal membrane would exist. Considering these permeation resistances separately, the prominent percentage ratio of $R_{m/g}$ to R_{total} for insulin (≥50%) revealed the substantial contribution of the mucous/glycocalyx layers to the whole process of insulin's permeation across the intestinal mucosa. The mucous/glycocalyx layers presumably would exist as an enzymatic barrier and/or diffusional barrier. In fact, some reports showed that the pancreatic enzymes capable of degrading insulin reside in the mucous/glycocalyx layers (5,24). Also, hydrophilic macromolecular compounds diffuse through mucous more slowly compared to hydrophilic low molecular weight compounds (25). However, the P_{app} of FD-4, a stable hydrophilic macromolecule with a comparable molecular weight to insulin, was not significantly different

between the PBS-treated and the hyaluronidase-treated groups, i.e., with and without the mucous/glycocalyx layers. In addition, transcellular diffusive resistance was unchanged, because the P_{app} of antipyrine, a transcellular transport marker, was not affected by the removal of the mucous/glycocalyx layers. Thus, as a diffusional barrier, the contribution of the mucous/glycocalyx layers to the whole diffusive resistance for hydrophilic macromolecules such as insulin from the mucosal side to the serosal side would be negligible compared to the intrinsic resistance of the intestinal membrane. On the other hand, with regard to the enzymatic barrier, significant degradation of insulin occurred in the rinse solution collected after the hyaluronidase pretreatment, which would be attributable to the removal of the preepithelial enzymatic barrier compartment, i.e., proteolytic activity of pancreatic enzymes enmeshed in the preepithelial layers. The resultant increase in the concentration slope of insulin across the membrane presumably attributed to an increase in the permeation of insulin. Therefore, it is clear that the mucous/glycocalyx layers affected insulin permeation predominantly as an enzymatic barrier and not as a diffusional barrier, irrespective of small intestinal regions.

In the present study, the apparent permeability of insulin in the PBS-pretreated group, i.e., in the presence of the mucous/glycocalyx layers, increased in the following order: duodenum < jejunum < ileum. This tendency in the increase in P_{app} of insulin toward distal small intestinal regions was consistent with those of previous *in vitro* insulin permeation studies using different small intestinal segments (17,18). Morishita *et al.* (26), using an *in situ* absorption study with different intestinal loops washed with saline, reported an obvious increase in insulin absorption in the ileum, the distal part of the small intestine. Assuming that the mucous/glycocalyx layers act predominantly as an enzymatic barrier to insulin permeation irrespective of small intestinal regions, the order of degradation half-life of insulin in the rinse solution collected after the hyaluronidase pretreatment (duodenum > jejunum > ileum; Table III) should correspond to the relative magnitude of enzymatic degradation activity in the mucous/glycocalyx layers in the small intestinal regions. The variation in degradation activity in the mucous/glycocalyx layers would be related to the amount of pancreatic enzymes enmeshed in the mucous/glycocalyx layers. In fact, the luminal concentrations of pancreatic enzymes decline toward the distal intestine (27). The pancreatic enzyme activity of trypsin and chymotrypsin in the duodenum decreases to almost one-half in the jejunum and to one-third in the ileum (28). On the other hand, only slight differences in the P_{app} of FD-4 existed among the small intestinal regions (Table II), which is consistent with a previous report showing that there were no significant differences between the P_{app} of FD-3 (FITC-dextran, average molecular weight of 3 kDa) in the jejunum and in the ileum (29). From this result, we deduce that there are no significant regional differences in the intrinsic permeation resistance of the intestinal membrane to insulin. Therefore, variation in enzymatic activity in the mucous/glycocalyx layers is one of the main factors that account for regional differences in the transport of insulin.

Meanwhile, although the molecular weight of insulin is similar to that of FD-4, the P_{app} of insulin in the hyaluronidase-pretreated group was still much lower than that of FD-4, indicating the existence of another enzymatic barrier other than that present in the mucous/glycocalyx layers. Insulin could be exposed to enzymes within the mucous/glycocalyx layers as well as the brush-border membrane region, which hyaluronidase pretreatment failed to remove, during its transport through the epithelial membrane. The brush-border membrane has various enzymes, whose type and distribution depend on location (30). While the enzymes comprising insulin degradation activity were not fully defined, indeed, the brush-border membrane has insulin-degrading activity (31,32). In addition, our previous study using the hyaluronidase-pretreated ileal loop with coadministration of a protease inhibitor indicated the existence of a brush-border membrane enzyme that acted as a degradation barrier to insulin permeation (16). In this study, the order of P_{app} of insulin in the hyaluronidase-pretreated group, duodenum < jejunum < ileum (Table II), would correspond to the relative magnitude of enzymatic degradation activity in the brush-border membrane among the small intestinal regions. Consequently, the relative magnitude of enzymatic degradation activity to insulin in not only the mucous/glycocalyx layers, but also in the brush-border membrane, tends to increase toward the upper small intestine. Thus, our technique allows comparison of the relative regional differences in enzymatic degradation activity in the mucous/glycocalyx layers and the brush-border membrane, and also their contribution to the permeation of insulin across the small intestinal membrane. In addition, further precise *in vitro* and/or *in situ* investigations of the entire enzymatic barrier, e.g., types and V_{max} and K_m of certain enzymes, might be promising. Currently, there are practical needs for the peroral delivery of enzymatic labile drugs such as insulin. Thus, we thought our technique would be helpful in designing a rational strategy to increase drug absorption toward therapeutically effective level by controlling resistance levels in the mucous/glycocalyx layers. It is especially important to consider the regional differences in degradation activity in both the mucous/glycocalyx layers and brush-border membrane, which is an obstacle for the transport of certain drugs across intestinal mucosa.

CONCLUSIONS

We demonstrated that hyaluronidase pretreatment immutably removed the mucous/glycocalyx layers without causing detectable damage to the membrane integrity irrespective of small intestinal regions, and that the mucous/glycocalyx layers contribute to insulin permeation predominantly as an enzymatic barrier and not as a diffusional barrier, irrespective of small intestinal regions. The enzymatic degradation activity to insulin in the mucous/glycocalyx layers tends to increase toward the upper small intestine in the following order: duodenum > jejunum > ileum. This would be one factor accounting for regional differences in the transport of insulin. The P_{app} of insulin in the hyaluronidase group was much lower than that of FD-4 and showed regional differences in the following order: duodenum < jejunum < ileum. This implies that regional differences exist in the enzymatic barrier to the transport of insulin in the brush-border membrane region. Consequently, our hyaluronidase pretreatment technique would be useful for investigation of the region-dependent contribution of the mucous/

glycocalyx layers to the transport of specific drugs across the small intestinal mucosa.

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