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Role of the mucous/glycocalyx layers in insulin permeation across the rat ileal membrane

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Abstract

The contribution of mucous/glycocalyx layers, as a diffusional or enzymatic barrier, to the absorption of insulin was investigated in situ and in vitro studies using rats. To remove the mucous/glycocalyx layers, ileal segments were exposed to a hyaluronidase solution in situ. The removal of the layers was confirmed by transmission electron microscopy, and the safety of the hyaluronidase pretreatment was established based on light microscopy, a constant mucosal membrane electrical resistance and the absence of lactate dehydrogenase leakage. In the in situ loop absorption experiment, hyaluronidase pretreatment significantly increased the plasma insulin level accompanied by an obvious hypoglycemic response. In the in vitro transport experiment, the apparent permeability coefficient of insulin was significantly increased by the hyaluronidase pretreatment, whereas that of 4.4 kDa fluorescein isothiocyanate-labeled dextran and of antipyrine, respective markers for passive para- and transcellular permeation, was unaffected. In the insulin degradation experiment in vitro, a significant amount of insulin was degraded in the compartment removed by hyaluronidase pretreatment. Thus, the mucous/glycocalyx layers functioned in insulin absorption as an enzymatic barrier and insignificantly affected diffusive absorption. In addition, co-administration of aprotinin, a protease inhibitor, further increased insulin absorption from ileum pretreated with hyaluronidase, implying the existence of another enzymatic barrier that influences insulin mucosal absorption.

Keywords: Insulin; Hyaluronidase; Mucous; Glycocalyx; Macromolecular drug delivery; Intestinal metabolism; Aprotinin

1. 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

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to parenteral administration, the oral bioavailability of these drugs is generally poor since multiple physical and enzymatic barriers are encountered within the gastrointestinal tract. In order to develop therapeutically effective oral dosage forms for peptides drugs, it is essential to understand which barrier(s) have important roles in peptide absorption and how they limit absorption from the intestine.

On top of the intestinal epithelium lie mucous and glycocalyx layers with a thicknesses of 5–10 and 0.1–0.5 μm, respectively (Ito, 1969; Pappenheimer, 2001). The layer of mucous consists of glycoproteins, enzymes, electrolytes and water, while the glycocalyx is primarily composed of glycoproteins, proteoglycans, and glycolipids including hyaluronan (Rambourg and Leblond, 1967; Woodley and Sterchi, 1978; Ugolev et al., 1979; Audus and Raub, 1993; Bai, 1994; Frey et al., 1996; Esko, 1999; Kovbasnjuk and Spring, 2000; Massey-Harroche, 2000). 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 (Frey et al., 1996; Varki, 1997). With regard to their contribution to the permeation of peptides across the intestinal mucosa, these layers have been considered as a diffusional barrier to the transport of peptides. 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 (Matthes et al., 1992; Larhed et al., 1998). On the other hand, it has been reported that these layers enmesh some endogenous constituents including pancreatic enzymes capable of degrading certain peptides (Ugolev et al., 1979). These observations imply that the mucous/glycocalyx layers contribute to peptide absorption as an enzymatic barrier and/or diffusion barrier.

In a previous study, we demonstrated that the ileal mucous/glycocalyx layers could be removed by hyaluronidase, an enzyme capable of reducing the glycocalyx component, without causing detectable cellular damage and thereby, significant roles for these layers in the absorption of insulin, chosen as a model peptide, from ileum could be identified in an in situ system (Morishita et al., 2004). We found that removal of the ileal mucous/glycocalyx layers resulted in a marked increase in the absorption of insulin, whereas diffusive absorption of macromolecular dextrans was unaffected. It was supposed that the mechanisms were associated with reduced proteolytic activity, however, the precise mechanisms remained to be fully substantiated. Therefore, the aim of this study was to prove that the mucous/glycocalyx layers functioned as enzymatic barriers to insulin absorption in in situ and in vitro permeation experiments. In this study, first we confirmed whether hyaluronidase pretreatment, modified so as to more closely resemble the in vivo application, would successfully remove the mucous/glycocalyx layers preserving the membrane's integrity. Then, the roles of the diminished mucous/glycocalyx layers in the metabolic loss of insulin during absorption were investigated directly. Furthermore, the influence of the layers on membrane permeability through the para- and transcellular pathway was evaluated using fluorescein isothiocyanate-labeled dextran with a weight-averaged molecular weight of 4.4 kDa (FD-4), a hydrophilic compound, and antipyrine, a lipophilic compound, as a marker for passive para- and transcellular permeation, respectively. In addition, aprotinin, a serine protease inhibitor, was used to prove the existence of another enzymatic barrier to insulin mucosal absorption other than the mucous/glycocalyx layers.

2. Materials and methods

2.1. Materials

Crystalline human recombinant insulin (USP; 28.6 IU/mg), fluorescein isothiocyanate-labeled dextran with a weight-averaged molecular weight (MW) of 4.4 kDa (FD-4), lyophilized hyaluronidase (EC 3.2.1.35; Type IV-S from bovine testes; MW = 56 kDa, 1320 U/mg solid), aprotinin (from bovine pancreas; 4 TIU/mg) and sodium taurodeoxycholate were purchased from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, MO, USA). Antipyrine was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other reagents were of analytical grade and used as received from the suppliers.

2.2. Hyaluronidase pretreatment and in situ absorption experiments

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 Co. Ltd. (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, then were fasted for 24 h prior to experiments. Following anesthetization with an intraperitoneal injection of sodium pentobarbital (50 mg/kg; Dainippon Pharmaceutical Co. Ltd., Osaka, Japan), rats were restrained in a supine position on a thermostatically controlled board at 37 °C.

Hyaluronidase pretreatment was carried out as previously described with a slight modification. The pretreatment condition, for example, the concentration of hyaluronidase and exposure time, were employed based on the condition optimized in the previous paper, which afforded maximum hypoglycemic effect by administration of insulin (Morishita et al., 2004). Briefly, the ileum was exposed following a small midline incision carefully made in the abdomen, and proximal-to-ileocecal junction segments (length = 10 cm) were cannulated at both ends using polypropylene tubing (4 mm o.d., 2 mm i.d., Saint-Gobain Norton Co. Ltd., Nagano, Japan). Subsequently, these segments were securely ligated to prevent fluid loss. The ileal segments 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. Then, the segments were carefully reinserted into the abdominal cavity and the abdominal wall was sutured to prevent heat loss. The PBS was composed of 137 mM NaCl, 2.6 mM KCl, 6.4 mM $Na_2HPO_4 \cdot 12H_2O$ and 1.4 mM KH_2PO_4. The dose of hyaluronidase was that having a maximum effect to completely remove mucous/glycocalyx layers in a previous study (Morishita et al., 2004). At the end of the exposure, the segments were gently rinsed with 20 mL of PBS at 37 °C. Before and after the pretreatment with hyaluronidase, the rinsed-out solutions were collected to determine the peptidolytic activity involved as described below. These rinsed-out solutions were centrifuged for 10 min (4 °C, 2700 × *g*), and supernatants were used for the peptidolytic degradation experiment.

In the in situ absorption experiment, 0.5 mL of insulin solution at 37 °C was directly administered into an ileal loop (6 cm) made from a pretreated segment (10 cm). Insulin was initially dissolved in 200 μL of 0.1 M HCl, then PBS was added to yield a concentration of 20 IU/mL; the solution's pH was simultaneously adjusted to 7.4 using 0.1 M NaOH. Thus, the dose of insulin was 50 IU/kg body weight. Blood samples (0.15 mL) were withdrawn from the jugular vein at 5 min prior to administration, and 5, 10, 15, 30, 60, 120, 180 and 240 min following administration. Additional intraperitoneal injections of sodium pentobarbital (12.5 mg/kg) were necessary every 1 h following the administration to maintain the anesthesia. Plasma was separated by centrifugation at 13,400 × *g* for 1 min and kept under refrigeration until the analysis. The plasma insulin levels were measured by immuno-chemiluminometric assay using a microplate luminometer (Mithras LB940, Beltold Japan Co. Ltd., Osaka, Japan). The total area under the insulin concentration curve ($[AUC]_{insulin}$) from time 0 to 4 h was estimated from the sum of successive trapezoids between each data point. Blood glucose levels were measured with a glucose meter (Novo Assist Plus, Novo Nordisk Pharma Ltd., Tokyo, Japan). These were described as a percentage of pre-dose glucose level and hence, as referenced with the corresponding blood glucose levels seen in the control (PBS-treated) group, the extent of hypoglycemic response was calculated as the area above the curve ($[AAC]_G$) for 0–4 h using the trapezoidal method.

In addition, an in situ absorption experiment was carried out with aprotinin, a serine protease inhibitor, to evaluate the contribution of another enzymatic barrier remaining after the mucus/glycocalyx layers had been removed by the hyaluronidase treatment, to the permeation of insulin across the ileal membrane. Aprotinin at 0.2, 0.5, 2 and 5 TIU/kg, was co-administered with insulin (50 IU/kg) into an ileal loop made from a pretreated segment.

2.3. Histological and biochemical examination of the ileal membranes following hyaluronidase pretreatment

The ileal segments were pretreated with PBS (control) or hyaluronidase in PBS as described above. Some experiments employed pretreatment with 1.0% (w/v) sodium taurodeoxycholate as an positive control

because of its known induction of mucosal damage (Ennis et al., 1990).

2.3.1. Light microscopy

The ileal segments were removed following the pretreatment and fixed with 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Subsequently, thin cross-sectional samples prepared with a microtome, were stained with hematoxylin-eosin (Kanto Chemical Co. Inc., Tokyo, Japan) for light microscopic observation to assess the tissue damage histologically.

2.3.2. Transmission electron microscopy (TEM)

The segments were removed from the body and fixed with 2.5% glutaraldehyde and 2.0% paraformaldehyde 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 Co. Ltd., Tokyo, Japan). Thin cross-sectional samples prepared with an ultramicrotome (MT-5000, Du Pont Co. Ltd., Wilmington, DE, USA) were finally stained with uranyl acetate and lead citrate to be examined by TEM (H-7500, Hitachi Co. Ltd., Tokyo, Japan) for evaluating the removal of the mucous/glycocalyx layers.

2.3.3. Lactate dehydrogenase (LDH) leakage

Following the pretreatment, the rinsed-out solutions were collected to determine the leakage of LDH, an intracellular enzyme often used to evaluate intracellular integrity (Swenson et al., 1994). LDH was quantified using 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.

2.4. Degradation of insulin in rinsed-out solutions

Insulin degradation experiments in the rinsed-out solutions collected before and after the pretreatment as described above were conducted to determine the peptidolytic activity of the mucous/glycocalyx layers removed by hyaluronidase. An insulin solution (600 μM) was prepared as described for the in situ absorption experiment. The solution (100 μL) and the rinsed-out solution (5 mL) were preequilibrated separately at 37 °C for 10 min. Just after the addition of the rinsed-out solution to the insulin solution, the mixture was vortexed for 2 s and then 100 μL of the mixture was immediately taken as the zero-time sample. The mixture was continuously incubated at 37 °C. At pre-determined 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 −20 °C until HPLC analyses were performed.

The insulin analysis was performed with a computer-controlled gradient high-pressure liquid chromatographic (HPLC) system (L-6000, Hitachi Co. Ltd., Tokyo, Japan). The gradient system used consisted of mobile phase A, water containing 0.1% trifluoroacetic acid (TFA) adjusted to pH 3 with phosphoric 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 mm × 4.6 mm) connected to a C8 precolumn. The gradient mobile phase was run at a flow rate of 1 mL/min. The UV–vis detector was set at 210 nm.

2.5. In vitro transport experiments

In vitro permeation experiments were performed with an Ussing chamber using the ileal membranes pretreated with either PBS (control) or hyaluronidase in PBS as described above. Following pretreatment, the ileal 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 in order to prevent the adsorption of insulin by the surface of the chamber and container. Krebs–Ringer's solution was composed of 108.0 mM NaCl, 11.5 mM D-glucose, 15.0 mM $NaHCO_3$, 4.7 mM KCl, 1.8 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 4.9 mM Na-glutamate, 5.4 mM Na_2-fumarate and 4.9 mM 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 Co. Ltd., 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, and then the ileal membranes were allowed to equilibrate for 20 min. 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 μM for insulin, 10 mg/mL for FD-4, and 4 mg/mL for antipyrine. The insulin solution was prepared as for the in situ absorption experiment using Krebs–Ringer's solution instead of PBS. 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 monitored simultaneously every 10 min during the experiment, and 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 ileal membranes mounted in the chamber.

The insulin concentration was measured by immuno-chemiluminometric assay using a microplate luminometer (Mithras LB940, Beltold Japan Co. Ltd., Osaka, Japan). The FD-4 concentration was determined using a microplate luminometer at excitation and emission wavelengths of 485 and 535 nm, respectively. The Antipyrine concentration was determined by the HPLC method, in which apparatus was the same as for the in vitro insulin degradation experiments. 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 C8 column (250 mm × 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-versus-time curve. The apparent permeability coefficient (P_{app}) of each compound was calculated as shown below:

$$P_{app} = \frac{J_{ss}}{C}$$

where C is the initial donor concentration.

2.6. Statistical analysis

Each value was expressed as the mean ± standard error (S.E.). Multiple ANOVA was performed, followed by the Dunnett method to compare the values for P_{app}. The values for P_{app} between the groups were compared using Student's unpaired t-test; p values < 0.05 were considered significant.

3. Results

3.1. In situ absorption of insulin from ileal segments pretreated with hyaluronidase

Fig. 1 shows (a) plasma insulin and (b) blood glucose levels following in situ administration of insulin at 50 IU/kg into the ileal segments pretreated with PBS (control) and hyaluronidase. While no apparent absorption of insulin or hypoglycemic response was ob-

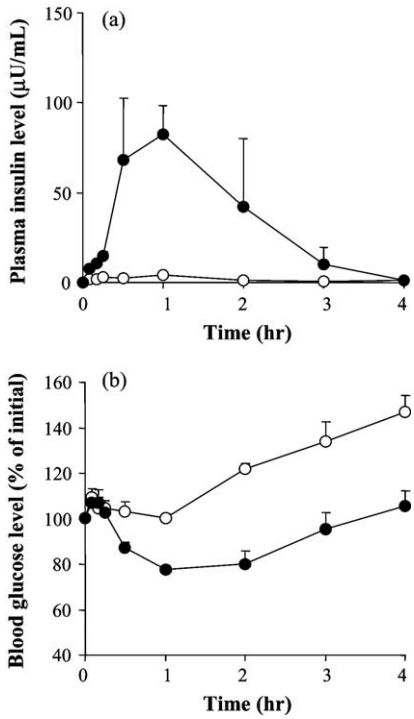

Fig. 1. (a) Plasma insulin level and (b) blood glucose level vs. time profiles following in situ administration of insulin (50 IU/kg) into ileal segments pretreated with PBS and hyaluronidase. Keys: (◯) PBS (control); (●) hyaluronidase at 192,000 U/mL. Data represent the mean ± S.E. from $n = 4$.

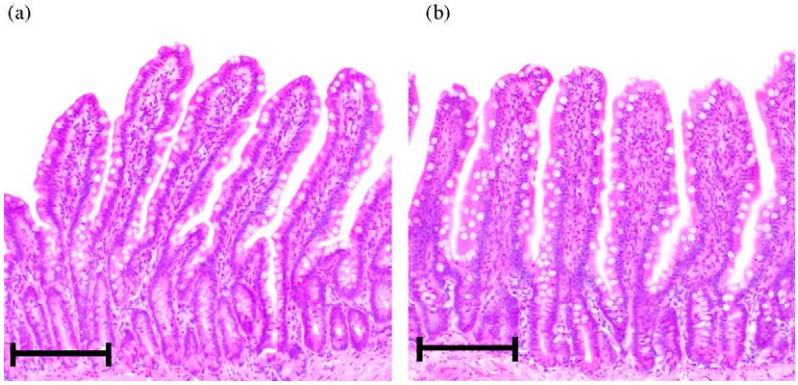

Fig. 2. Light micrographs of ileal mucosal membranes pretreated with (a) PBS and (b) hyaluronidase at 192,000 U/mL (×200 of magnification). The bars indicate 100 μm. Tissues were stained with hematoxylin and eosin following fixation using glutaraldehyde (2.5%) and paraformaldehyde (2.0%).

served in the control group, a significant increase in the plasma insulin level accompanied by an obvious hypoglycemic response was observed in the hyaluronidase-treated group. These results were consistent with our observation (Morishita et al., 2004) and verified directly that hyaluronidase pretreatment significantly increased ileal absorption of insulin.

3.2. Histological, biochemical and electrophysiological examinations of ileal membranes following hyaluronidase pretreatment

Histological micrographs of the ileal mucosal membranes pretreated with (a) PBS and (b) hyaluronidase are shown in Fig. 2. No apparent histological damage was found in the hyaluronidase-treated mucosal membranes and cells, compared to the PBS-treated (control) counterparts. The amount of LDH that leaked into the mucosal lumen was negligible even following the hyaluronidase pretreatment, which was similar to the leakage seen in the PBS-treated (control) group, while the leakage was dramatically induced with 1.0% (w/v) sodium taurodeoxycholate (Table 1). Likewise, regardless of the hyaluronidase pretreatment, the values for R_m remained constant throughout the course of the experiment (≈35 Ω cm^2; data not shown). These results

Table 1
Lactate dehydrogenase (LDH) leakage following PBS (control), hyaluronidase and 1.0% (w/v) sodium taurodeoxycholate pretreatment

Pretreatment	LDH leakage [U]
Control	1.0 ± 0.6
Hyaluronidase	1.2 ± 0.4
Sodium taurodeoxycholate	12.3 ± 1.6*

Data: mean ± S.E. (n = 3–4).
* $p < 0.05$ against control.

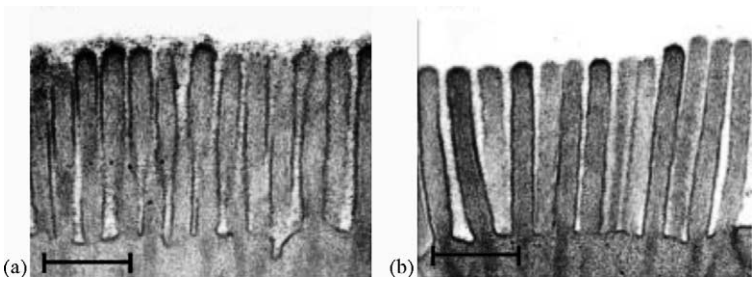

Fig. 3. Electron micrographs of ileal mucosal membranes pretreated with (a) PBS and (b) hyaluronidase at 192,000 U/mL (×30,000 of magnification). The bars indicate 0.5 μm. Tissues were stained with uranyl acetate and lead citrate following primary and secondary fixations using glutaraldehyde (2.5%), paraformaldehyde (2.0%) and osmium tetroxide (1.0%).

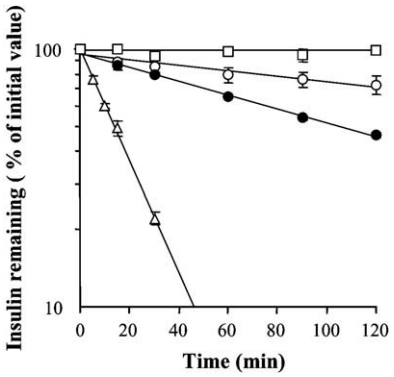

Fig. 4. Degradation profile of insulin as a function of time in the rinsed-out solutions. Keys: (□) PBS solution (control), (◯) PBS pretreatment, (●) hyaluronidase pretreatment, and (ρ) before pretreatment (10 times dilution). Data represent the mean ± S.E. from $n = 3$ to 4.

suggested that the ileal membrane integrity and cellular tight junctions remained mostly unaltered by the pretreatment with hyaluronidase for 30 min.

Fig. 3 shows electron micrographs of (a) PBS-treated (control) and (b) hyaluronidase-treated ileal mucosal membranes. The mucous layer was removed via chemical fixations and dehydration of the ileal segments and thus, unobserved in any specimens. In the control group, a glycocalyx layer approximately 70–100 nm thick was clearly observed as electron-dense layers, enveloping the top of the ileal microvillus (Fig. 3a). In contrast, this layer was removed from the hyaluronidase-treated ileal membranes, which had a near-naked microvillus (Fig. 3b). Meanwhile, as observed in the micrographs on the right shown in Fig. 2, the epithelial cellular integrity seemed to be unaffected by the hyaluronidase treatment. Therefore, it was likely that the loss of the mucous/glycocalyx layers caused, in part, the increased absorption of insulin, resulting in significant hypoglycemic effects.

3.3. Stability of insulin in the rinsed-out solution

Fig. 4 shows profiles of the degradation of insulin in the rinsed-out solution collected before or after the pretreatment. In the rinsed-out solution collected before the pretreatment, even though the solution was diluted 10-fold, insulin was quickly degraded. This implies that extensive proteolytic degradation occurred in the intestinal tract in quite a short time. On the other hand, as we hypothesized, a substantial amount of insulin was degraded in the rinsed-out 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 rinsed-out solution collected after the hyaluronidase pretreatment was significantly higher than that in the solution collected after PBS pretreatment (111 min versus 387 min for hyaluronidase- versus PBS-treated group; $p < 0.05$). These results suggest that insulin was extensively degraded in the compartment removed by hyaluronidase pretreatment.

3.4. In vitro transport experiment

Fig. 5 and Table 2 show the transport profiles and the apparent permeability coefficient (P_{app}) of (a) insulin, (b) FD-4, and (c) antipyrine across the ileal mucosal membranes pretreated with PBS and hyaluronidase. The mean P_{app} values of insulin was $(0.358 \pm 0.051) \times 10^{-8}$ cm s^{-1} (Table 2). This value was smaller than those reported P_{app} values of insulin from rat ileum values of $(6.82 \pm 1.87) \times 10^{-7}$ cm s^{-1} (Schilling and Mitra, 1990) and $(1.05 \pm 0.21) \times 10^{-6}$ cm s^{-1} (Asada et al., 1995). The variation in permeability coefficients could be explained by differences in apparatus, tissue preparation, concentrations of insulin in donor side studied, analytical method employed and the duration of experiments.

In the case of insulin, the P_{app} value observed in the hyaluronidase-treated group was four times higher than that of the control group. In contrast, the transport profiles of FD-4 and antipyrine across the ileal membrane exhibited no significant differences irrespective of pretreatment with PBS or hyaluronidase. The ratio of

Table 2
Apparent permeability coefficient of insulin, FD-4 and antipyrine during transport across the rat ileum with or without hyaluronidase pretreatment

Solute	Apparent permeability, P_{app} (10^{-8}cm/s)		Ratio
	PBS	Hyaluronidase	
Insulin	0.358 ± 0.051	1.429 ± 0.072*	4.0
FD-4	6.144 ± 0.354	6.004 ± 0.605	1.0
Antipyrine	264.8 ± 27.1	272.0 ± 15.8	0.9

Data: mean ± S.E. ($n = 4$–7).

* $p < 0.05$ against control. There were no significant differences in the groups of FD-4 and antipyrine.

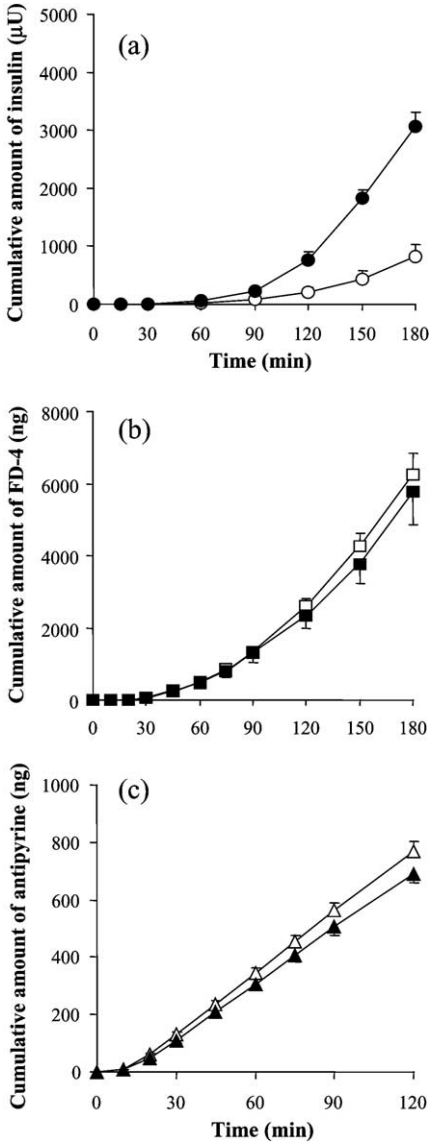

Fig. 5. Time course of (a) insulin, (b) FD-4, and (c) antipyrine transport across rat ileal segments pretreated with PBS and hyaluronidase at 192,000 U/mL. Keys: PBS (open symbol), hyaluronidase (closed symbol); (◯, ●) insulin; (□, ■) FD-4; (△, ▲) antipyrine. Data represent the mean ± S.E. from $n = 4$ to 7.

P_{app} (hyaluronidase-treated)/P_{app} (PBS-treated) values of these drugs were almost 1 (Table 2).

3.5. *In situ absorption experiment with aprotinin*

Fig. 6 shows the effects of aprotinin on (a) plasma insulin levels and (b) blood glucose levels following

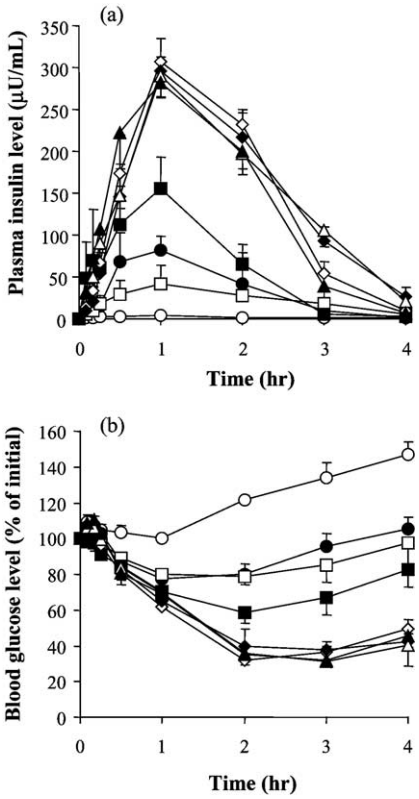

Fig. 6. (a) Plasma insulin level and (b) blood glucose level vs. time profiles following in situ administration of insulin (50 IU/kg) in the presence of aprotinin into the ileal segments pretreated with PBS and hyaluronidase at 192,000 U/mL. Keys: PBS (open symbol), hyaluronidase (closed symbol); (◯, ●) 0 TIU/kg (control); (□, ■) 0.2 TIU/kg; (◊, ♦) 2.0 TIU/kg; (△, ▲) 5.0 TIU/kg. Data represent the mean ± S.E. from $n = 4$.

the in situ administration of insulin into the ileal segments pretreated with PBS and hyaluronidase. Their $[AUC]_{insulin}$ and $[AAC]_G$ values are shown in Table 3. In the hyaluronidase-treated group, a low dose of aprotinin further enhanced the absorption of insulin from the ileum, but the effect appeared to be saturated at the higher dose: the values for both $[AUC]_{insulin}$ and $[AAC]_G$ differed insignificantly at 2 and 5 TIU/kg. Likewise, in the PBS-treated pretreatment group, aprotinin enhanced insulin absorption but the dose required was higher than in the hyaluronidase-treated group. However, at a higher aprotinin dose (>2 TIU/kg), there were no significant differences in $[AUC]_{insulin}$ and $[AAC]_G$ values between control and hyaluronidase-treated groups.

Table 3
$[AUC]_{insulin}$ and $[AAC]_G$ following the ileal administration of insulin with aprotinin after hyaluronidase pretreatment

Aprotinin dose (TIU/kg)	Pretreatment	$[AUC]_{insulin}$ (μU/mL h)	$[AAC]_G$ (% glu. reduc. h)
0.0	PBS	7 ± 3 *	5.2 ± 2.5 *
	Hyaluronidase	145 ± 26	51.9 ± 12.9
0.2	PBS	95 ± 30 *	60.6 ± 22.0 *
	Hyaluronidase	250 ± 47	112.6 ± 21.9
2.0	PBS	602 ± 34	188.4 ± 8.4
	Hyaluronidase	610 ± 55	180.0 ± 10.5
5.0	PBS	607 ± 28	192.4 ± 15.5
	Hyaluronidase	562 ± 48	191.3 ± 10.7

Data: mean ± S.E. ($n = 4$).
* $p < 0.05$ against PBS pretreatment.

4. Discussion

We have already demonstrated in a previous study that the hyaluronidase pretreatment successfully removed the mucous/glycocalyx layers of the ileal epithelium without causing detectable damage to the membrane integrity using a hyaluronidase perfusion method (Morishita et al., 2004). The results of the present study clearly showed efficacy and safety of hyaluronidase pretreatment, using a method of exposure that more closely resembled the in vivo application, based on microscopic observations alongside the values for LDH leakage and R_m (Figs. 2 and 3, Table 1). Therefore, the hyaluronidase exposure seemed to be useful for identifying the roles of the mucous/glycocalyx layers in drug absorption similar to the situation in vivo.

The increase in insulin absorption from the ileal membrane pretreated with hyaluronidase could be clearly demonstrated by measuring the plasma insulin concentration, coupled with the blood glucose level, which were used as surrogate measures of insulin absorption in the previous study (Morishita et al., 2004). As mechanisms behind the increase in insulin absorption following hyaluronidase pretreatment, the mucous/glycocalyx layers presumably contribute to insulin absorption as an enzymatic barrier and/or diffusion barrier. With regard to the enzymatic barrier, the existence of pancreatic enzymes enmeshed in the mucous/glycocalyx layers was demonstrated by Ugolev, who separated the glycocalyx layer was by means of a technique using an agar replica (Ugolev et al., 1979). Therefore, it is considered that the proteolytic activity pancreatic enzymes enmeshed in the pre-epithelial layers could be reduced with the removal of the mucous/glycocalyx layers. In fact, a significant amount of insulin was degraded in the compartment removed by hyaluronidase pretreatment (Fig. 4). This is the first direct demonstration in vivo that the ileal epithelial mucous/glycocalyx layers participate in the degradation of insulin.

In the in vitro permeation experiment, the contribution of mucous/glycocalyx layers to insulin absorption was further substantiated by comparing the permeation behavior of insulin with that of FD-4 and antipyrine, para- and trans-cellular permeation markers, respectively. Consequently, irrespective of the presence or absence of the mucous/glycocalyx layers, the permeation behavior of FD-4 was unchanged (Fig. 5, Table 2), and this phenomenon was consistent with our previous observation. These results were probably due to the much lower intrinsic epithelial membrane permeability for macromolecules of 4.4 kDa, compared to that for diffusion through the mucous/glycocalyx layers. However, although a much smaller and lipophilic compound, antipyrine appeared to be unaffected in its transport by the hyaluronidase pretreatment, and indeed, the values of the apparent permeability coefficient shown in Table 2 were not significantly different between the PBS-treated and the hyaluronidase-treated groups. Thus, these results imply that the ileal mucous/glycocalyx layers are an insignificant impediment to diffusive para- and trans-cellular absorption across the ileal membranes. On the other hand, as opposed to the permeation behavior of FD-4 and antipyrine, that of insulin was clearly changed by hyaluronidase

pretreatment (Fig. 5, Table 2). Since significant degradation of insulin occurred in the rinsed-out solution collected after the hyaluronidase pretreatment, the change in permeation was presumably attributed to the increase in the concentration slope of insulin across the ileal membrane due to removal of the pre-epithelial enzymatic barrier compartment. Therefore, it is clear that the mucous/glycocalyx layers influence insulin absorption predominantly as an enzymatic barrier not a diffusional barrier. In addition, although the molecular size of insulin was not so much different from that of FD-4, the apparent permeability coefficient values of insulin in hyaluronidase-pretreated group were still lower than that of FD-4. This implies the existence of another enzymatic barrier unable to be removed by the hyaluronidase pretreatment, which coincided with the results of in situ absorption experiment with aprotinin as ascribed below.

Aprotinin is a bovine pancreatic kallikrein inhibitor with a molecular mass of 6.5 kDa. Several reports have shown that the co-administration of aprotinin leads to an increased bioavailability of peptide and protein drugs including insulin, due to its inhibitory effect on trypsin as well as chymotrypsin (Morishita et al., 1993; Bernkop-Schnurch, 1998; Uchiyama et al., 1999). Since the intestinal mucosal toxicity of aprotinin was minor, as evaluated by the leakage of Evans blue from the systemic circulation (Uchiyama et al., 1999), the influence of aprotinin on the diffusional barrier would be insignificant. Therefore, the effect of aprotinin on the increase of peptide and protein drug absorption may be predominantly based on the inhibition of proteolytic activity. In this study, we showed that aprotinin further enhanced insulin absorption from the ileum pretreated with hyaluronidase, as indicated by $[AUC]_{insulin}$ and $[AAC]_G$ (Fig. 6 and Table 3), suggesting the existence of another enzymatic barrier, which was probably unable to be removed by the hyaluronidase pretreatment. During its transport through the epithelial membrane, insulin could be exposed to enzymes within the mucous/glycocalyx layers as well as the brush-border membrane region. According to a study on the degradation of insulin in subcellular compartments, the brush-border membrane has insulin-degrading activity (Bai, 1995). The brush-border membrane has various enzymes, the type and distribution of which depend on species and site (Woodley, 1994). A few brush-border membrane enzymes capable of degrading insulin, such as insulin degrading enzyme (IDE), have been identified (Chang and Bai, 1996). It was reported that IDE is distributed in various tissues and plays an important role in insulin degradation (Kuo et al., 1993). Although IDE mainly resides in the cytosol, this enzyme also would be present on the brush-border membrane (Chang and Bai, 1996; Chang et al., 1997). On the other hand, it has been reported that aprotinin was capable of inhibiting pure IDE activity (Roth et al., 1985). Based on these findings, the fact that IDE was inhibited by aprotinin, might account for substantial influence of brush-border membrane enzymes on insulin permeation. However, the contribution of IDE to the brush-border membrane's enzymatic barrier function remains to be fully substantiated.

For drug absorption studies, the human colonic adenocarcinoma-derived cell line Caco-2 is the most frequently used. This cell line is well accepted as a model to investigate the relationship between the molecular structure, physicochemical properties, and absorption potential of drugs (Conradi et al., 1991; Artursson and Karlsson, 1991). However, cultured epithelial cell layers lack a variety of different cell types; such as goblet cells, therefore they do not produce mucin molecules forming a mucous layer. From the viewpoint of the contribution of enzymatic/diffusional barriers in the mucous/glycocalyx layers to drug permeation, the lack of these barriers in the cultured epithelial cell layers might lead to the constitutional difference in the permeation behavior between conditions in vitro and in vivo (in physiological state), especially in the case of drug substances susceptible to enzymatic degradation in the mucous/glycocalyx layers. The hyaluronidase pretreatment method used in the present study is unique in the sense that the role of the mucous/glycocalyx in drug absorption could be identified, mimicking in the situation in vivo. In addition, the method would be useful for evaluating the role of the mucous/glycocalyx in regulating the access and bioadhesion of various pharmaceutical carriers to the intestinal mucosal membranes.

5. Conclusions

We have demonstrated that hyaluronidase pretreatment for 30 min was removed the mucous/glycocalyx

layers of the ileal epithelium without causing detectable damage to intra- and intercellular integrity. This separation technique revealed that these layers do not impede diffusive para- and transcellular absorption across the ileal membranes, but significantly influence the absorption of insulin, predominantly as an enzymatic barrier. In fact, it was proved that a significant amount of insulin was degraded in the compartment removed by hyaluronidase pretreatment. Moreover, co-administration of aprotinin in addition to the hyaluronidase pretreatment increased insulin absorption, suggesting the existence of brush-border membrane enzymes that influence the permeation of insulin, although the substantiality of these enzymatic barriers remain to be fully investigated.

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