Role of the Mucous/Glycocalyx Layers in Absorption of Insulin from Small Intestine

Yoshinobu Aoki

CONTENTS

List of Publication

1. *In situ* ileal absorption of insulin in rats: effects of hyaluronidase pretreatment diminishing the mucous/glycocalyx layers: Mariko Morishita, Yoshinobu Aoki, Masahiro Sakagami, Tsuneji Nagai, and Kozo Takayama. *Pharm. Res.* 21:309-316 (2004). <presented in Chapter 1 of this dissertation>

2. Role of the mucous/glycocalyx layers in insulin permeation across the rat ileal membrane: Yoshinobu Aoki, Mariko Morishita, and Kozo Takayama. *Int. J. Pharm.* 297 98-109 (2005). <presented in Chapter 1 and 2 of this dissertation>

3. Region-dependent role of the mucous/glycocalyx layers in insulin permeation across rat small intestinal membrane: Yoshinobu Aoki, Mariko Morishita, Kazunori Asai, Bunshiro Akikusa, Shuji Hosoda, and Kozo Takayama *Pharm. Res.* 22:1854-1862 (2005). <presented in Chapter 3 of this dissertation>

Abbreviations

AAC	area above the curve
AB	alcian blue
AUC	area under the concentration-time curve
BBME	brush border membrane enzyme
Cmax	maximum concentration
FDs	fluorescein isothiocyanate-labeled dextrans
FD-4	fluorescein isothiocyanate-labeled dextran with molecular weights of 4.4 kDa
FD-20	fluorescein isothiocyanate-labeled dextran with molecular weights of 20 kDa
FD-40	fluorescein isothiocyanate-labeled dextran with molecular weights of 40 kDa
FITC	fluorescein isothiocyanate
HE	hematoxylin eosin
HPLC	high performance liquid chromatography
IDE	insulin degrading enzyme
Isc	short circuit current
Jss	steady-state flux
LDH	lactate dehydrogenase
LM	light microscopy
Papp	apparent permeability coefficients
PBS	phosphate-buffered saline, pH 7.4
PD	potential difference
Rm	membrane electrical resistance
R_{mem}	apparent permeation resistance in the mucosal membrane

$R_{m/g}$	apparent permeation resistance in the mucous/glycocalyx layers
R_{total}	apparent total permeation resistance
TEM	transmission electron microscopy
Tmax	the time to reach the Cmax

GENERAL 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. However, most these peptide drugs are administered by the parenteral route that is often painful, difficult, and occasionally dangerous. Aside from the so-called alternative routes of application such as the nasal and transdermal routes [1,2], there is no doubt that the oral route is the most favored, because it offers the greatest ease of application.

Insulin, a 51 amino acid peptide, has been greatly used to treat patients with diabetes which requires parenteral administration mainly by the s.c. routes. Endogenous insulin is secreted by the pancreas into tributaries of hepatic portal vein, resulting in direct delivery of insulin to the liver, which is the principal target organ of insulin and also removes half of the insulin presented to it in a single transhepatic circulation [3]. This suggested that oral insulin therapy is thought to be close to physiological state, since it may offer a means of improving portal levels of insulin and may also be curtail the peripheral hyper insulinemia. Therefore, insulin will be one of the drugs desired for orally administration.

In order to gain sufficient blood concentrations after mucosal application of peptide and/or protein drugs such as insulin, various barriers have to be overcome. These barriers include the enzymatic barrier and diffusional barrier. The enzymatic barrier is based on secreted and membrane bound peptidases. On the other hand, the diffusional barrier consists of permeation resistances of the mucous/glycocalyx layers and the intrinsic intestinal membrane.

In past decade, to overcome these enzymatic barrier and diffusional barrier, several *in vivo* and *in vitro* studies using enzyme inhibitors and absorption enhancers have been conducted and they have shown the enhanced macromolecular absorption [4-9], however,

most of these studies were aimed at the enhancement and hence, unable to locate cellular and/or subcellular barrier compartments kinetically responsible for controlling such macromolecular behavior. Therefore, for rational development of therapeutically effective oral dosage forms for peptide drugs such as insulin, 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.

Meanwhile, recent evidence has suggested that the mucous/glycocalyx layers, extracellular domains directly attached to the intestinal epithelium, could function significantly as barrier to certain amino acids, nutrients and macromolecules, even compared to the intestinal epithelium [10,11]. Nevertheless, the information regarding these layers and specifically, polypeptidic molecules (i.e., insulin) is scarce and thus, the layers are under-exploited to fully understand their roles and functions in these macromolecular behaviors, which should be differentiated from those of other kinetic barrier compartments (i.e., intestinal epithelium). Therefore, in this study, to elucidate that the mucous/glycocalyx layers impeded the absorption of insulin from small intestine, the absorption of insulin using rat ileum following diminishment of mucous/glycocalyx layers was examined compared with the absorption behavior of fluorescein isothiocyanate-labeled dextrans (FDs), stable non-peptide hydrophilic macromolecules. The mucous/glycocalyx layers could be removed by an *in situ* pretreatment technique using hyaluronidase, the enzyme degrading hyaluronan, one of components of the mucous/glycocalyx layers.

The mucous/glycocalyx layers have been thought to function as diffusional barrier and/or enzymatic barrier to the absorption of insulin. However, it is unclear which diffusional or enzymatic barrier of mucous/glycocalyx layers predominantly contributes to

whole permeation process of insulin across the intestinal mucosa. Therefore, to elucidate this point, the degradation behavior and the permeation behavior of insulin in the components of the mucous/glycocalyx layers were examined. On the other hand, besides the enzymatic barrier of the mucous/glycocalyx layers, the brush border membrane enzymes (BBME) have been thought to function as enzymatic barrier to the absorption of insulin [12,13]. Thus, it was examined whether BBME affected to the absorption of insulin.

It has been reported that the absorption of insulin differs among various intestinal regions [6,14,15], implying the varied impedance of the mucous/glycocalyx layers to the absorption of insulin among different small intestinal regions. Therefore, to elucidate the regional differences in the contribution of the mucous/glycocalyx layers to the absorption of insulin as a diffusional and/or enzymatic barrier, the absorption of insulin using rat duodenum, jejunum and ileum following diminishment of mucous/glycocalyx layers was examined.

In Chapter 1, the impedance of the mucous/glycocalyx layers to the absorption of insulin from rat ileum was investigated, focusing on the comparison with the absorption behavior of FDs, and confirmation of the successful diminishment of the mucous/glycocalyx layers by the hyaluronidase pretreatment. In Chapter 2, the contribution of mucous/glycocalyx layers to the absorption of insulin as a diffusional and/or enzymatic barrier was investigated. Also, the existence of BBME as enzymatic barrier to the absorption of insulin was investigated. In Chapter 3, the regional differences in the contribution of the mucous/glycocalyx layers to the absorption of insulin as a diffusional and/or enzymatic barrier were investigated.

CHAPTER 1

In Situ Ileal Absorption of Insulin: Effects of Hyaluronidase Pretreatment Diminishing the Mucous/Glycocalyx Layers

1. Introduction

The intestinal mucous and glycocalyx layers have been known to envelop the absorptive surface of the brush border with their thicknesses of 5-10 and 0.1-0.5 μm, respectively [10,16]. These layers are believed to protect apical cell surface against microbial pathogens and foreign materials partially by virtue of electrical repulsion for negatively charged sugar moieties [11,17]. Moreover, it has also been shown that the layers function as size-selective barriers to prevent some molecules and particles from gaining access to the surface receptors and enzymes [11,17,18]. Specifically, the intestinal glycocalyx is primarily composed of glycoproteins, proteoglycans and glycolipids including hyaluronan and thereby, enmeshes some endogenous constituents including pancreatic enzymes and ectopeptidases that are capable of degrading certain amino acids, nutrients.

Studies have been conducted in other (lung and kidney) epithelial systems as well as endothelial systems where the transfer of macromolecular solutes and genes was impeded by the presence of the glycocalyx [18-21]. Notably, these were identified in the experimental systems where enzymes like hyaluronidase and/or neuraminidase were pretreated to diminish the glycocalyx layers [18-21]. Therefore, on the hypothesis that the intestinal (ileal) mucous/glycocalyx layers could be similarly diminished by hyaluronidase pretreatment, successful identification of the significant roles in the absorption of certain macromolecular i.e. insulin and/or proteolysis were expected.

In this chapter, it was investigated that the mucous/glycocalyx layers impeded the absorption of insulin from rat ileum following diminishment of the mucous/glycocalyx layers by hyaluronidase pretreatment, compared to the absorption of differently sized, fluorescein isothiocyanate-labeled (FITC) dextrans (FDs: FD-4, FD-20 and FD-40), as non-peptide hydrophilic macromolecules. The diminished mucous/glycocalyx layers by the hyaluronidase

pretreatment was ensured by microscopic observation, while intra- and inter-cellular integrity and/or damage were examined histologically, biochemically, and electrophysiologically.

2. Materials and methods

2.1. Materials

Crystalline bovine insulin (USP; 28.2 IU/mg), fluorescein isothiocyanate-labeled dextrans with weight-averaged molecular weights (MW) of 4.4, 20 and 40 kDa (FD-4, FD-20 and FD-40, respectively), lyophilized hyaluronidase (EC 3.2.1.35; Type IV-S from bovine testes; MW = 56 kDa, 1320 U/mg solid) and sodium taurodeoxycholate were purchased from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, MO, USA). All other reagents were of analytical grade and used as received from the suppliers.

2.2. Hyaluronidase pretreatment and *in situ* absorption experiment

This research was performed, complied with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals at Hoshi University. Male Wistar rats weighing 180-220 g were purchased from Sankyo Lab Service Co., Ltd. (Tokyo, Japan). While animals were housed in rooms controlled between 23 ± 1 °C and 55 ± 5 % relative humidity and allowed free access to water and food during acclimatization, they were fasted for 24 hr prior to experiments. Following anesthetization by 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. The ileum was exposed following small midline incision carefully made in the abdomen, and its proximal-to-ileocecal junction segments (length = 10 cm) were cannulated at their both ends using polypropylene tubings (4 mm o.d., 2 mm i.d., Saint-Gobain Norton Co., Ltd., Nagano, Japan). Subsequently, these were securely ligated to prevent fluid loss and carefully returned to their original location inside the peritoneal cavity.

Initially, hyaluronidase was pretreated to the ileal segments via "perfusion" to efficiently diminish the mucous/glycocalyx layers. Therefore, as its methodological validation,

transmission electron microscopy (TEM) and light microscopy (LM) were employed to ensure their diminishment, while histological, biochemical and electrophysiological examinations were to evaluate possible membrane damages due to the pretreatment. Phosphate buffered saline (PBS; pH 7.4; control) or various concentrations (80, 160, 320 and 480 U/mL) of hyaluronidase in PBS at 37°C were singly circulated via "perfusion" through the cannula at 1.0 mL/min for 30 min using a peristaltic pump (MP-3, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). It is noted that PBS (in mM) was composed of 137 NaCl, 2.6 KCl, 6.4 $Na_2HPO_4 \cdot 12H_2O$ and 1.4 KH_2PO_4. Following the "perfusion", the cannulation tubings were removed and the ileal segments were tightly closed; approximately 1 mL of the perfusion solutions remained left in the segments. The rats were further left on the board at 37°C for 1 hr to be recovered from the elevated blood glucose level due to the surgical operation described above.

In the *in situ* absorption experiments, 0.5 mL of insulin or each of FDs solutions (FD-4, FD-20 and FD-40) at 37°C was directly administered into an ileal loop (6 cm) made from the pretreated segment (10 cm). Insulin was initially dissolved in 200 μL of 0.1 M HCl, followed by a dilution with PBS to yield its concentration at 20 IU/mL; the pH of solution was simultaneously adjusted to 7.4 using 0.1 M NaOH. Each of FDs solutions (FD-4, FD-20 and FD-40) was prepared at 5 mg/mL in PBS. Thus, the doses of insulin and each of FDs were 50 IU/kg and 5 mg/kg body weight, respectively. 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 at every 1 hr following administration to maintain the anesthesia. Blood glucose levels were measured with a glucose meter (Novo Assist Plus, Novo Nordisk Pharma Ltd., Tokyo, Japan) in insulin studies and used as surrogate measures of insulin absorption. These were described as % 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 hr using the trapezoidal method. In contrast, the absorption of FDs was evaluated from the plasma concentrations determined by fluorometry at excitation and emission wavelengths of 490 and 520 nm, respectively (F-4010, Hitachi Co., Ltd., Tokyo, Japan), following blood centrifugation at 13400 g for 2 min at room temperature. While the maximum concentration (C_{max}) and the time to reach the C_{max} (T_{max}) were visually obtained from the plasma concentration profiles, the area under the curve ($[AUC]_{FD}$) of the profiles was calculated for 0-4 hr following administration using the trapezoidal method.

Insulin absorption was further studied in the same *in situ* system described above, but using the ileal segments pretreated with hyaluronidase via "exposure". This study was designed to explore the feasibility of increasing ileal absorption of insulin by hyaluronidase administered in a way that is realistically applicable to *in vivo*. The ileal segments were exposed to 1.0 mL (37°C) of PBS (pH 7.4; control) or hyaluronidase in PBS at 9600, 19200, 48000, 96000, 192000 and 384000 U/mL for 30 min, followed by tight closures at their both ends; no perfusion was employed. At the end of the exposure, the segments were gently rinsed with 20 mL of PBS at 37°C and subsequently, *in situ* insulin's absorption experiments at a dose of 50 IU/kg were performed, as described earlier.

2.3. Transmission electron microscopy

The ileal segments were pretreated with PBS (control) or hyaluronidase in PBS via "perfusion" or "exposure", as described above. The segments were removed from the body and fixed with 2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). A secondary fixation employed 1.0 % osmium tetroxide in the same buffer for 1 hr, followed by dehydration and embedding in Epon 812 (NISSIN-EM Co., Ltd., Tokyo, Japan).

Thin cross-sectional samples were prepared by ultramicrotome (MT-5000, Du Pont Co., Ltd., Wilmington, DE, USA), which were finally stained with uranyl acetate and lead citrate to be examined by TEM (H-7500, Hitachi Co., Ltd., Tokyo, Japan) for evaluating the diminishment of the mucous/glycocalyx layers.

2.4. Histological, biochemical and electrophysiological examination of the ileal membranes

The ileal segments were pretreated with PBS (control) or hyaluronidase in PBS via "perfusion" or "exposure", as described above. Some experiments employed pretreatment with 1.0 % (w/v) sodium taurodeoxycholate as a positive control due to its known induction of mucosal damage [22].

Light microscopy

The intestinal segments were removed following the pretreatment and fixed with 2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Subsequently, thin cross-sectional samples were prepared by the microtome, followed by staining for light microscopic observation. Samples were stained with hematoxylin-eosin (HE) stain and with alcian-blue (AB) stain at pH 2.5 (Kanto Chemical Co., Inc., Tokyo, Japan) to assess tissue damages histologically and to assess the diminishment of the mucous/glycocalyx layers, respectively [23].

Lactate dehydrogenase (LDH) leakage

Following the pretreatment, the perfused solutions were collected to determine the leakage of LDH, an intracellular enzyme often used to evaluate intracellular integrity [24]. LDH was quantified using LDH-Test Wako (Wako Pure Chemical Industries, Ltd., Osaka,

Japan) following in-house validation that had ensured the lack of assay interference by the presence of hyaluronidase in the sample solutions.

Ileal membrane electrical resistance (Rm)

Because membrane electrophysiological parameters like Rm are not possible to be determined directly in the *in situ* system described above, these were alternatively studied using *in vitro* Ussing chamber where the ileal membranes pretreated with either PBS or 320 U/mL hyaluronidase in the *in situ* system were mounted. Following the 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). 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, and the (flat sheet) membranes were mounted in an Ussing chamber (CEZ-9100, Nihon-Kohden Tokyo Co., Ltd., Tokyo, Japan) maintained at 37°C. Apical and basal compartments were filled with 5.0 mL of Krebs-Ringer's solution and stirred with oxygenation (95% O_2/5% CO_2) for 20 min prior to monitoring electrophysiological parameters. The spontaneous transmucosal potential difference (PD) and the short circuit current (Isc) were recorded simultaneously at every 10 min for 90 min, and the values for Rm were calculated by PD/Isc, based on the 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 identical bathing solutions, yet in the absence of ileal membranes mounted in the chamber.

2.5. Statistical analysis

Each value was expressed as mean ± standard error (SE). Multiple ANOVA was performed, followed by Dunnett method to compare the values for $[AAC]_G$. The values for T_{max}, C_{max} and $[AUC]_{FD}$ between the groups were compared by Student's unpaired *t*-test; p values < 0.05 were considered significant.

3. Results

3.1. Insulin and FDs absorption from the ileal segments pretreated by hyaluronidase

Figure 1 shows the blood glucose levels following *in situ* administration of insulin at 50 IU/kg into the ileal segments pretreated with PBS (control) and various concentrations of hyaluronidase via "perfusion". The hyaluronidase pretreatment unaffected the blood glucose levels without insulin administration (data not shown). No apparent hypoglycemic response was observed in the control group, demonstrating the absence of appreciable insulin absorption from the PBS-treated ileal segments (Fig. 1). In contrast, hyaluronidase pretreatment at concentrations ≥ 160 U/mL caused significant hypoglycemic responses, suggesting the increased insulin absorption by virtue of hyaluronidase pretreatment. It should be noted however, that the effects appeared to reach the maximum at the higher

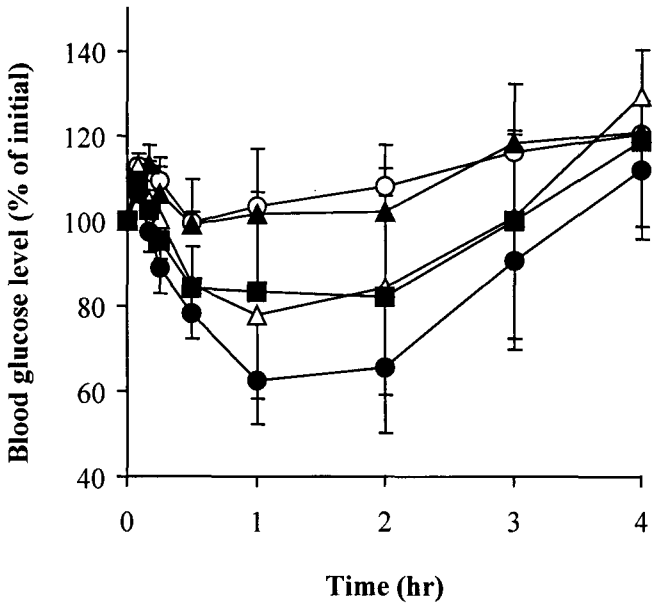

Figure 1. Blood glucose level versus time profiles following *in situ* administration of insulin (50 IU/kg) into the ileal segments pretreated with PBS and various concentrations of hyaluronidase via "perfusion".

Each data represents mean ± SE from n = 4. Key: (○) PBS (control); (▲, △, ● and ■) hyaluronidase at 80, 160, 320 and 480 U/mL, respectively.

concentrations; the values for $[AAC]_G$ differed insignificantly at 320 and 480 U/mL (95.7 ± 40.4 and 74.5 ± 47.9 % glucose reduction·hr, respectively). Meanwhile, the absorption of insulin appeared to be terminated at ≤ 3 hr (Fig. 1), implying substantial the degradation of insulin in the ileal loop and/or relatively short periods of the hyaluronidase effects via "perfusion" on this increased insulin absorption.

Table 1 shows pharmacokinetic parameters (C_{max}, T_{max} and $[AUC]_{FD}$) for each of FDs derived from the plasma concentration vs. time profiles following *in situ* administration in the absence (PBS) and presence of 320 U/mL hyaluronidase "perfusion" pretreatment; the latter caused the increased insulin absorption, as shown in Fig. 1. In contrast to the case of insulin case, plasma concentration profiles of any FDs appeared to be unaffected by the hyaluronidase pretreatment (the profiles not shown) and indeed, any kinetic parameters shown in Table 1 were not significantly different between the PBS-treated (control) and the hyaluronidase-treated groups.

Table 1. Pharmacokinetic Parameters Derived from the Plasma Concentration vs. Time Profiles for FDs; FD-4, FD-20 and FD-40, Following *in situ* Administration into the Ileal Segments Pretreated with PBS (Control) and 320 U/mL Hyaluronidase via "Perfusion".

Solute	Pretreatment	C_{max} [μg/mL]	T_{max} [hr]	$[AUC]_{FD}$ [μg/mL·hr]
FD-4	Control	0.14 ± 0.04	2.9 ± 0.8	0.28 ± 0.06
	Hyaluronidase	0.20 ± 0.01	3.7 ± 0.3	0.31 ± 0.02
FD-20	Control	0.04 ± 0.01	2.3 ± 1.0	0.04 ± 0.02
	Hyaluronidase	0.02 ± 0.01	1.3 ± 0.9	0.05 ± 0.03
FD-40	Control	0.04 ± 0.01	1.3 ± 0.9	0.10 ± 0.04
	Hyaluronidase	0.05 ± 0.02	0.9 ± 0.4	0.13 ± 0.07

Data: mean ± SE (n = 3-4)

C_{max}: the maximum concentration; T_{max}: the time to reach the C_{max}; $[AUC]_{FD}$: the area under the curve. There were no significant differences in any parameters between the groups.

3.2. Ileal mucous/glycocalyx layers following hyaluronidase pretreatment

Figures 2 and 3 show light micrographs (AB stain) and electron micrographs of the PBS-treated and the hyaluronidase (320 U/mL)-treated ileal mucosal membranes. From light microscopic observations (AB stain), intense staining along the apical side of the epithelial membranes was clearly observed for the PBS-treated (control) group, indicating the existence of the glycocalyx. In contrast, such a layer was hardly observed for the hyaluronidase-treated group; 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.

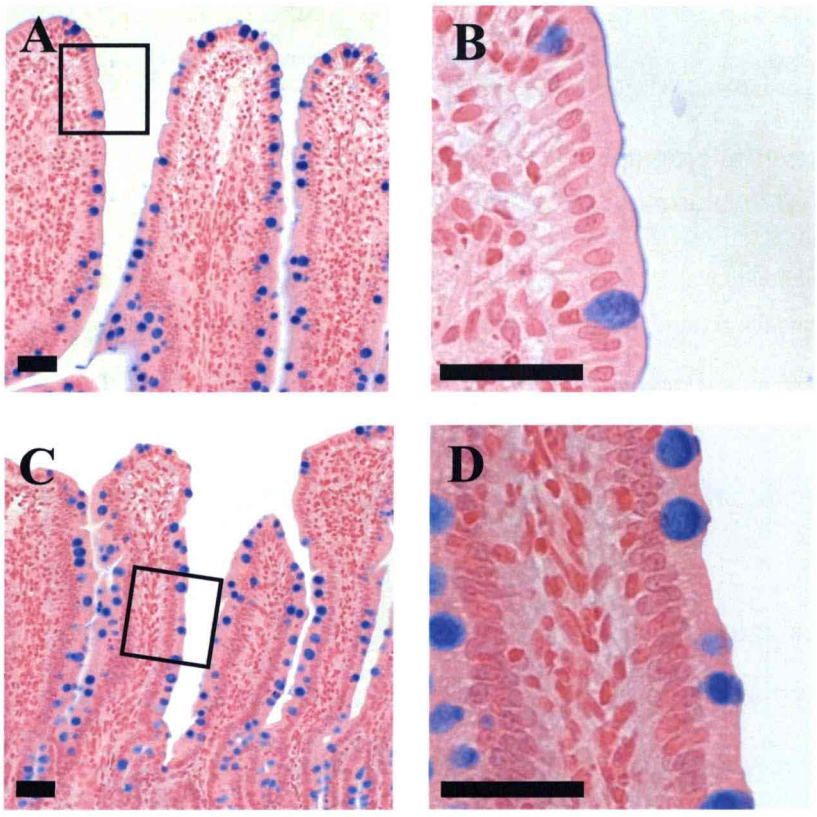

Figure 2. Light micrographs of ileal mucosa pretreated with PBS (A and B) and hyaluronidase at 320 U/mL (C and D) via "perfusion": (A and C; x 10, B and D; x 40).

The scale bar (thick bar) was 40 μm. Tissues were stained with AB (pH 2.5) following fixation using glutaraldehyde (2.5 %) and paraformaldehyde (2.0 %).

From the electron microscopic observations, approximately 70-100 nm thickness of the glycocalyx layer was clearly observed as electron-dense layers, enveloping the microvillus consistently for PBS-treated (control) group. In contrast, the hyaluronidase pretreatment was found to diminish such a layer remarkably, resulting in a near-naked microvillus.

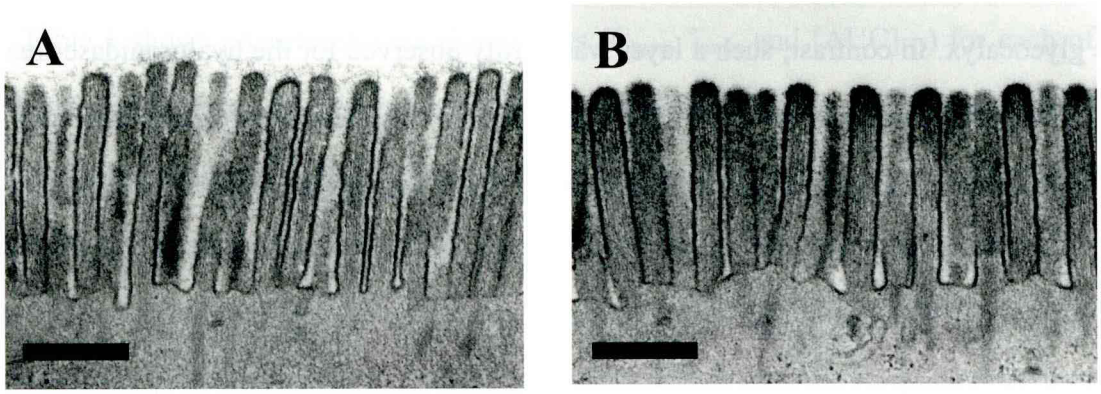

Figure 3. Electron micrographs of the ileal mucosal membranes pretreated with (A) PBS and (B) 320 U/mL hyaluronidase via "perfusion".

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 %), paraformaldehyde (2.0 %), and osmium tetroxide (1.0 %).

3.3. Histological, biochemical and electrophysiological examination of the ileal membranes

Histological micrographs (HE stain) of the ileal mucosal membranes pretreated with (A) PBS and (B) 320 U/mL hyaluronidase via "perfusion" are shown in Fig. 4. As observed in the electron micrographs, no apparent histological damages were found in the hyaluronidase-treated mucosal membranes and cells, compared to those in the PBS-treated (control) counterparts (Figs. 4-B and 4-A, respectively).

Table 2 shows the LDH leakage and the values for Rm in the absence (PBS; control) and presence of 320 U/mL hyaluronidase "perfusion" pretreatment. LDH was negligibly

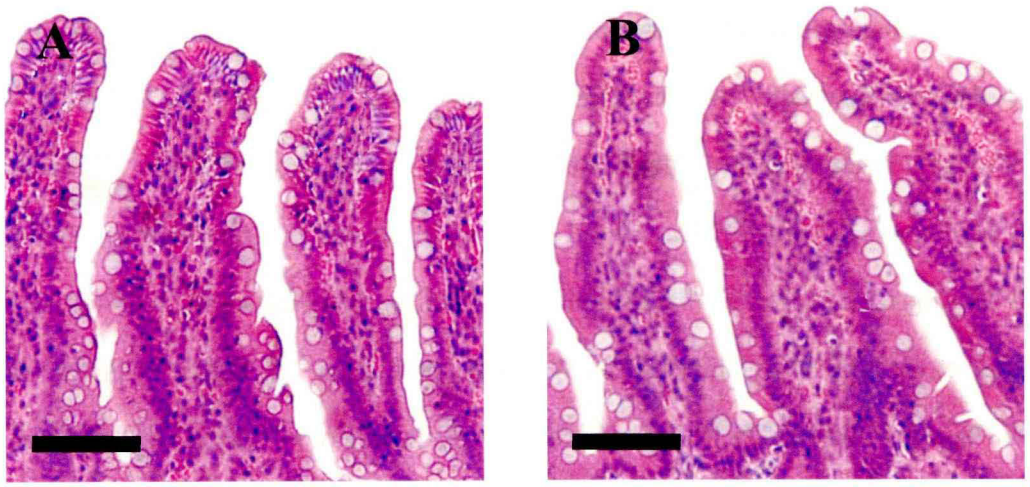

Figure 4. Light micrographs of the ileal mucosa pretreated with (A) PBS and (B) 320 U/mL hyaluronidase via "perfusion".

The scale bar (thick bar) was 100 μm. Tissues were stained with HE following fixation using glutaraldehyde (2.5 %), paraformaldehyde (2.0 %).

leaked into the mucosal lumen even following the hyaluronidase pretreatment, which was similar to the leakage seen in the PBS-treated group (1.1 ± 0.4 vs. 0.6 ± 0.1 U), while the leakage was dramatically induced with 1.0 % (w/v) sodium taurodeoxycholate (11.0 ± 2.5 U). Likewise, regardless of the presence or absence of the hyaluronidase pretreatment, the values for Rm remained constant throughout the course of the experiment (≈ 25 $\Omega \cdot cm^2$)

Table 2. LDH Leakage and Rm Following PBS (Control), 320 U/mL Hyaluronidase and 1.0 % (w/v) Sodium Taurodeoxycholate Pretreatment via "Perfusion"

Pretreatment	LDH leakage [U]	Rm [$\Omega \cdot cm^2$]	
		0 min	90 min
Control	0.6 ± 0.1	26.8 ± 0.9	24.3 ± 0.6
Hyaluronidase	1.1 ± 0.4	27.1 ± 1.2	25.3 ± 1.5
Sodium taurodeoxycholate	11.0 ± 2.5 *	ND	ND

Data: mean ± SE (n = 4). * $p < 0.05$ against control. ND: Not determined. The values for Rm differed insignificantly between the PBS-treated (control) and the hyaluronidase-treated groups across 90 min.

3.4. Insulin absorption and diminishment of mucous/glycocalyx layers following hyaluronidase "exposure" pretreatment

Although hyaluronidase "perfusion" at 320 U/mL successfully diminished the mucous/glycocalyx layers (Fig. 2, 3) and thereby, increased insulin absorption (Fig. 1), the method was an experimental manipulation and unlikely to be applicable to increase the absorption *in vivo*. Thus, hyaluronidase was pretreated in an "exposure" fashion, mimicking *in vivo* application where hyaluronidase is administered prior to macromolecular administration. Figure 5 shows the effects of such "exposure" pretreatments at various concentrations of hyaluronidase on (A) the blood glucose level and (B) the hypoglycemic response ($[AAC]_G$) following *in situ* administration of insulin at 50 IU/kg. Although much higher concentrations were necessary, compared to "perfusion", significant hypoglycemic

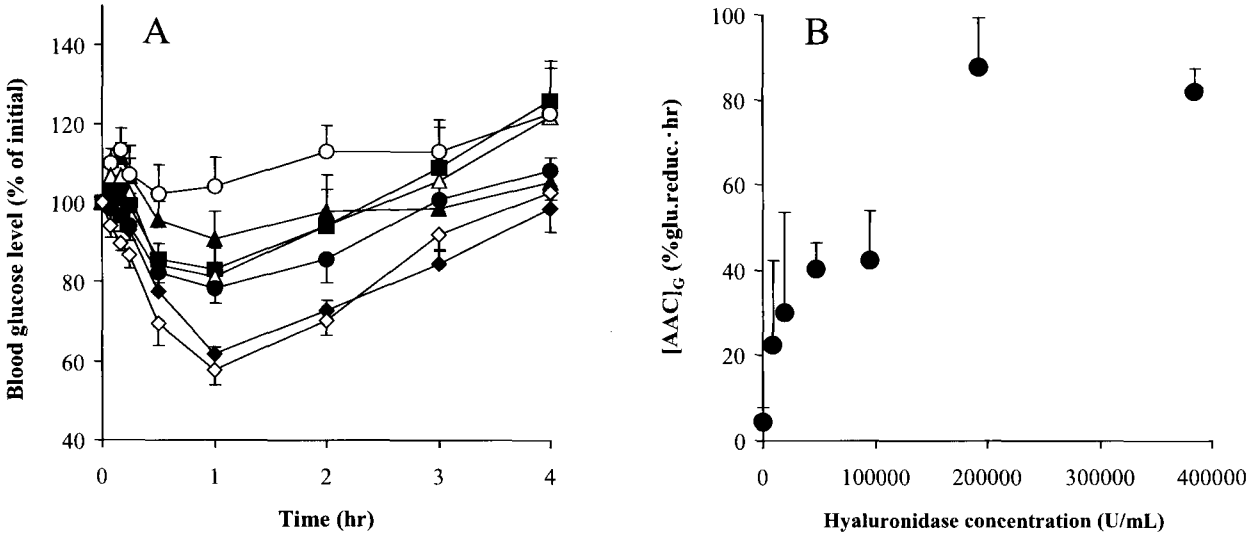

Figure 5. (A) Blood glucose level versus time profiles following *in situ* administration of insulin (50 IU/kg) into the ileal segments pretreated with PBS and various concentrations of hyaluronidase via "exposure".

Key: (○) PBS (control); (●, △, ■, ▲, ◆ and ◇) hyaluronidase at 9600, 19200, 48000, 96000, 192000 and 384000 U/mL, respectively. (B) The calculated $[AAC]_G$ versus hyaluronidase-pretreated concentration via "exposure". Each data represents mean ± SE from n = 4.

responses were observed via "exposure" at hyaluronidase concentrations ≥ 19,200 U/mL (Fig. 5-A). It appeared furthermore, that the enhancing effects were dose-dependent at concentrations ≤ 192,000 U/mL, while reaching the maximum of $[AAC]_G$ = 87.8 ± 11.6 % glucose reduction·hr (Fig. 5-B).

The successful diminishment of the mucous/glycocalyx layers by hyaluronidase pretreatment were confirmed by the light microscopic observation (AB stain) (Fig. 6) and the electron microscopic observation (Fig. 7) along with the case of pretreatment via "perfusion".

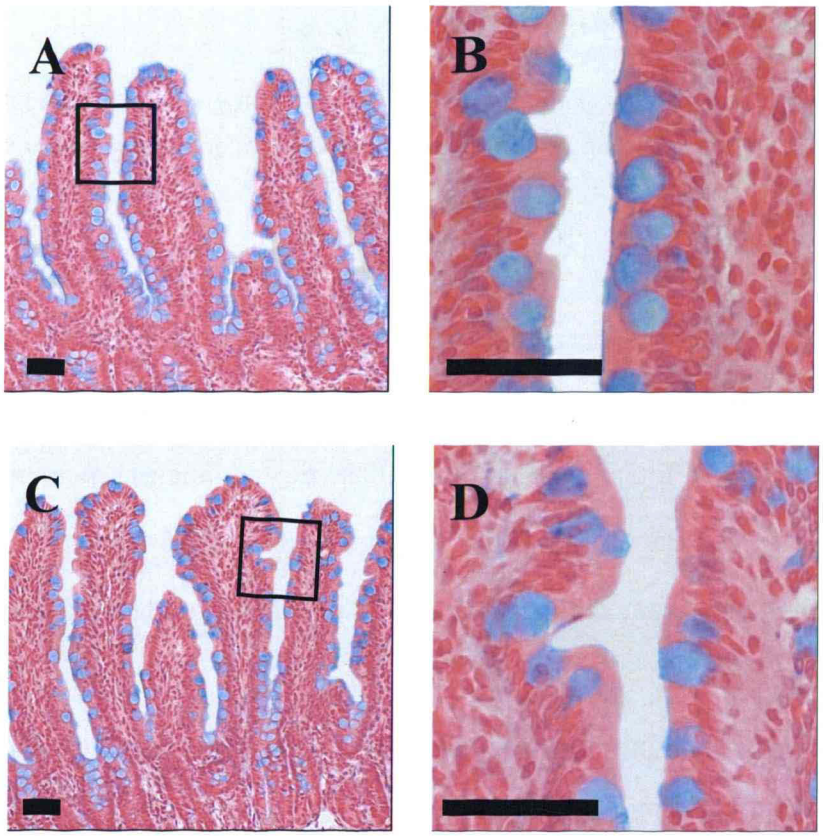

Figure 6. Light micrographs of ileal mucosa pretreated with PBS (A and B) and hyaluronidase at 192,000 U/mL (C and D) via "exposure": (A and C; x 10, B and D; x 40).

The scale bar (thick bar) was 40 μm. Tissues were stained with AB (pH 2.5) following fixation using glutaraldehyde (2.5 %) and paraformaldehyde (2.0 %).

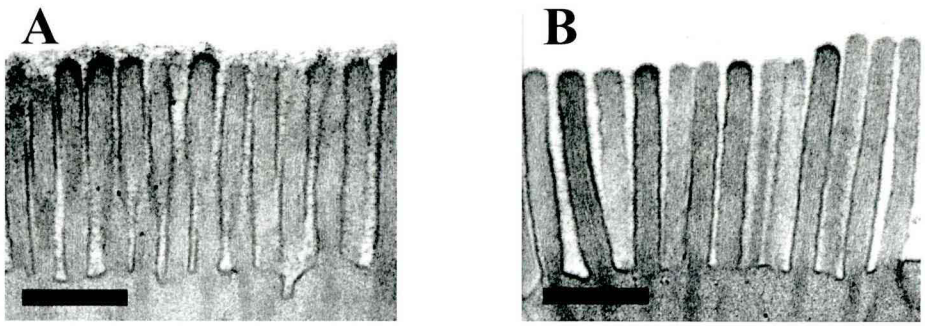

Figure 7. Electron micrographs of the ileal mucosal membranes pretreated with (A) PBS and (B) 192,000 U/mL hyaluronidase via "exposure".

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 %), paraformaldehyde (2.0 %) and osmium tetroxide (1.0 %).

Despite the use of much higher hyaluronidase concentrations (9,600-38,4000 U/mL), compared to "perfusion", as shown in LM micrograph (HE stain) in Fig. 8, no apparent histological damages were found in the hyaluronidase-treated mucosal membranes and cells, compared to those in the PBS-treated (control) counterparts (Figs. 8-B and 8-A, respectively). Also, there were no significant changes in LDH leakage between the PBS-exposure and the hyaluronidase-exposure; 1.0 ± 0.6 vs. 1.2 ± 0.3 U for control vs. 192,000 U/mL hyaluronidase (Table 3), while the leakage was dramatically induced with 1.0 % (w/v) sodium taurodeoxycholate (12.3 ± 1.6 U). Likewise, regardless of the presence or absence of the hyaluronidase pretreatment, the values for Rm remained constant throughout the course of the experiment (≈ 35 $\Omega \cdot cm^2$)

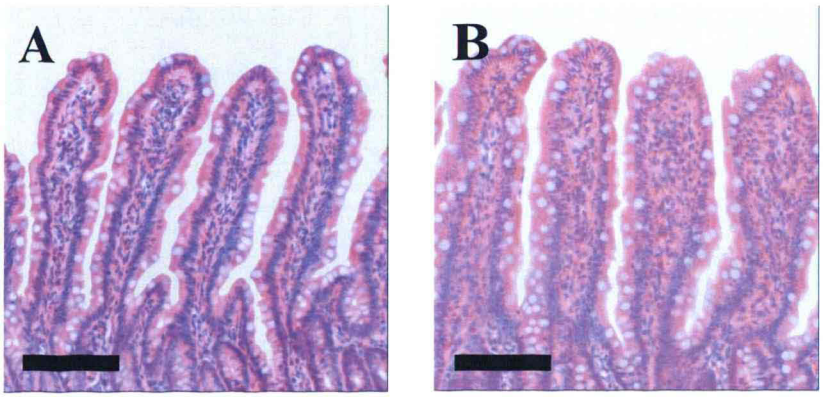

Figure 8. Light micrographs of the ileal mucosa pretreated with (A) PBS and (B) 192,000 U/mL hyaluronidase via "exposure".

The scale bar (thick bar) was 100 μm. Tissues were stained with HE following fixation using glutaraldehyde (2.5 %) and paraformaldehyde (2.0 %).

Table 3. LDH Leakage and Rm Following PBS (Control), 192000 U/mL Hyaluronidase and 1.0 % (w/v) Sodium Taurodeoxycholate Pretreatment via "Exposure"

Pretreatment	LDH leakage [U]	Rm [$\Omega \cdot cm^2$]	
		0 min	90 min
Control	1.0 ± 0.6	35.4 ± 2.2	37.1 ± 1.6
Hyaluronidase	1.2 ± 0.4	36.4 ± 2.1	37.0 ± 2.2
Sodium taurodeoxycholate	12.3 ± 1.6 *	ND	ND

Data: mean ± SE (n = 4). * $p < 0.05$ against control. ND: Not determined. The values for Rm differed insignificantly between the PBS-treated (control) and the hyaluronidase-treated groups across 90 min.

4. Discussion

Hyaluronidase pretreatment successfully diminished the mucous/glycocalyx layers of the ileal epithelium (Fig. 2, 3, 6, and 7) and thereby, induced significant hypoglycemic responses following insulin administration into the *in situ* ileal lumens (Figs. 1 and 5). In contrast, the absorption of non-peptide hydrophilic macromolecules (FDs: FD-4, FD-20 and FD-40) was not enhanced following the hyaluronidase pretreatment at a concentration (320 U/mL) via "perfusion" causing the increased absorption of insulin (Fig. 1 and Table 1). The microscopic observation alongside the values for LDH leakage and Rm showed that the membrane integrity and the cellular tight junctions appeared to remain undamaged (Fig. 3 and 7, Table 2 and 3). This is the first demonstration that the ileal epithelial mucous/glycocalyx layers played a significant role, specifically in the absorption of insulin, but not FDs (non-peptide hydrophilic macromolecules; polysaccharide), and such increased absorption of insulin was not caused by the cellular damage. Moreover, it has been shown that both "perfusion" and "exposure" pretreatments were effective to increase the absorption of insulin, and the magnitudes of the effect appeared to depend on hyaluronidase concentration (Figs. 1 and 5).

Unaltered absorption of FDs (Table 1) reasonably implies that the ileal mucous/glycocalyx layers existed as an insignificant impediment in diffusive absorption of macromolecules $\geq$ 4.4 kDa across the ileal membranes. This was presumably due to much lower intrinsic epithelial membrane permeability for this size of macromolecules, compared to diffusive counterpart through the mucous/glycocalyx layers. Unchanged values for LDH leakage and Rm (Table 3) following hyaluronidase pretreatment lend strong support to these results of FDs. Thus, the mechanisms of the increased absorption of insulin (Figs 1 and 5) would not be associated with facilitation of intra- and inter-cellular diffusion. Meanwhile, several authors have speculated active absorption of insulin through receptor-mediated

mechanisms [7,25,26], and in such case, the increased absorption of insulin could be caused by the increased accessibility to such transporting receptors in the absence of the glycocalyx. In fact, it has been reported that the glycocalyx played a significant role in certain ligand accessibility to the receptors present on the apical membranes of the intestinal epithelium, especially when its transport controlled overall kinetics [10,11,16]. Nevertheless, this possibility is unlikely, considering the extremely high insulin concentration (20 IU/mL) administered in the ileal segments, as opposed to relatively low Km values generally seen in those literatures [27,28].

Another possibility of the increased absorption of insulin shown in Figs. 1 and 5 is associated with the reduced proteolytic activity by virtue of the diminished mucous/glycocalyx layers by the hyaluronidase pretreatment. The hyaluronidase pretreatment diminished the mucous/glycocalyx layers (Fig. 2, 3, 6 and 7) and increased insulin absorption (Figs. 1 and 5), suggesting the important roles of hyaluronidase-removable enzymes in metabolic loss of insulin during absorption. Indeed, it has been shown that insulin was degraded by pancreatic proteases like trypsin and chymotrypsin, which are believed to reside in both intestinal fluids and the mucous/glycocalyx layers [29,30]. In this case, enzymes in the latter component could be removed as a result of the diminished mucous/glycocalyx layers by the hyaluronidase treatment. Moreover, the importance of exopeptidases (i.e. aminopeptidases and dipeptidylpeptidases) contributing to the metabolic loss of certain polypeptides including insulin has been suggested recently [12,31-33]. It is known that these enzymes are generally anchored with the epithelium and otherwise, floated as soluble forms [12,29-34] and thus, the latter was potentially removable by the hyaluronidase treatment. Notably, the increased hypoglycemic response following *in situ* insulin administration appeared to reach the similar maximum $[AAC]_G$ via both "perfusion" and "exposure" pretreatment of hyaluronidase (at 320 and 192,000 U/mL, respectively; Figs. 1 and 5). In

contrast, Morishita et al have shown previously [6], that aprotinin, which is known to be a trypsin and chymotrypsin inhibitor, induced further higher hypoglycemic responses ($[AAC]_G$ = 180.3 ± 29.3 glucose reduction·hr), compared to these maximum $[AAC]_G$ seen in this study, under the identical *in situ* system. Therefore, this further implied that the membrane-anchored proteolytic enzymes, which were probably unable to be removed by the hyaluronidase treatment, participated metabolic loss of insulin following administration. Thus, as first proposed by Ugolev and Laey for relatively small polypeptides [35], it is likely that the intestinal membranes equip multi-layers of enzymatic barriers to digest macromolecules including insulin, although kinetic contributions and mechanisms of each barrier function remain to be fully substantiated.

The results of the hyaluronidase "perfusion" pretreatment are quite unique in a sense that the mucous/glycocalyx layers could be identified as an important barrier for polypeptidic insulin. Classical approaches using absorption enhancers and/or enzyme inhibitors have primarily aimed at increasing intestinal absorption of insulin in both *in vivo* and *in vitro* systems [4-9] and hence, not specific enough to identify such subcellular barrier compartments kinetically responsible for creating impediments and their physiological regulation. In the latter aspect, the mucous/glycocalyx layers were thought to be reformed within a relatively short periods after hyaluronidase pretreatment as their physiological regulation. In fact, the intestinal glycocalyx has shown to be replaced constantly by newly synthesized glycocalyx components, based on acetate-3H monitoring [16]. Also, the turnover of intestinal mucous within short period was reported [36]. Therefore, assuming that mucous/glycocalyx layers would not be a diffusional barrier but an enzymatic barrier to insulin absorption, once the mucous/glycocalyx layers are removed by hyaluronidase pretreatment, the enzymatic barrier component against insulin in the reproduced mucous/glycocalyx layers would decline. In this point, the results from this "exposure" pretreatment would provide the feasibility of *in vivo*

application of hyaluronidase pre-administration in order to increase insulin absorption following oral administration without causing detectable cellular damage. Especially, for mucoadhesive devise for insulin oral delivery, the reformed mucous/glycocalyx layers after hyaluronidase pretreatment would be comfortable, since the inherent mucous/glycocalyx layers for such mucoadhesive devise were important as component to be adhered but obstructive to insulin absorption as an enzymatic barrier.

5. Conclusions

The impedance of the mucous/glycocalyx layers to the absorption of insulin from rat ileum following diminishment of the mucous/glycocalyx layers by hyaluronidase pretreatment was evaluated, compared to the absorption of FDs as non-peptide hydrophilic macromolecules. Successful diminishment of the mucous/glycocalyx layers of the ileal epithelium without causing detectable damage in intra- and inter-cellular integrity was enable by the hyaluronidase pretreatment via both "perfusion" and "exposure". As a result, it was found that the absorption of insulin was increased dramatically following diminishment of the mucous/glycocalyx layers, whereas those of FDs were unaffected.

Clearly, it was demonstrated that the mucous/glycocalyx layers played a significant role in ileal absorption of insulin, but not FDs.

CHAPTER 2

Substantial Barrier Property of the Mucous/Glycocalyx Layers to Insulin Permeation across the Ileal Mucosa

1. Introduction

As described in Chapter 1, the mucous/glycocalyx layers impeded the absorption of insulin from rat ileum. On the other hand, it was reported that the *in vitro* diffusion of hydrophilic macromolecules through the mucus were much slower than that of low molecular drugs [37], and also that the pancreatic enzymes capable of degrading insulin would reside in the mucous/glycocalyx layers [30]. These implied the mucous/glycocalyx layers would function as diffusional barrier and/or enzymatic barrier to the absorption of insulin. From the viewpoint of a rational strategy to increase the absorption of insulin toward therapeutically effective level by controlling resistance levels in the mucous/glycocalyx layers, it is desirable to elucidate these substantial barrier properties of the mucous/glycocalyx layers. Therefore, it was examined which diffusional or enzymatic barrier in the mucous/glycocalyx layers predominantly contributes to whole permeation process of insulin across the intestinal mucosa. The successful pretreatment technique using hyaluronidase allowed the examinations of the degradation behavior of insulin in the mucous/glycocalyx layers and the examinations of the permeation behavior of insulin across the ileal tissue with and/or without mucous/glycocalyx layers compared to the passive para- and transcellular permeation markers. Meanwhile, there were some reports that BBME has insulin-degrading activity, besides the enzymatic barrier of the mucous/glycocalyx layers [12,13]. Thus, it was examined whether BBME affected as enzymatic barrier to the absorption of insulin.

In this chapter, the *in vitro* permeation behavior of insulin across the ileal mucosa with and/or without mucous/glycocalyx layers was investigated, compared to FD-4, a stable non-peptide hydrophilic macromolecules with a comparable molecular weight to insulin, and antipyrine, a lipophilic compound, which were selected as passive para- and transcellular permeation markers, respectively. The *in vitro* degradation behavior of insulin in the

mucous/glycocalyx layers component was examined. The contribution of BBME to the absorption of insulin was investigated by *in situ* co-administration of the inhibitor against BBME.

2. Materials and Methods

2.1. Materials

Crystalline human recombinant insulin (USP; 28.6 IU/mg) and aprotinin (from bovine pancreas; 4 TIU/mg) were purchased from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, MO, USA). FD-4 and hyaluronidase were the same one in Chapter 1. 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. *In situ* hyaluronidase pretreatment

Hyaluronidase pretreatment via "exposure" was carried out as described in Chapter 1. The pretreatment condition, e.g. the concentration of hyaluronidase (192,000 U/mL) and exposure time (30 min), was selected based on the condition afforded maximum hypoglycemic effect by following administration of insulin and the successful diminishment of the mucous/glycocalyx layers. Before and after the pretreatment with hyaluronidase, the rinse solutions were collected to determine the peptidolytic activity involved as described below. These rinse solutions were centrifuged for 10 min (4°C, 2700 g), and supernatants were used for the peptidolytic degradation experiment.

2.3. 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. The insulin solution (600 μM) was prepared by the same procedure as described for the *in situ* absorption study in Chapter 1. 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 -80°C until HPLC analysis. HPLC analysis was performed with a computer-controlled gradient HPLC system (L-6000, Hitachi Co., Ltd., 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 mm x 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.

2.4. *In vitro* permeation experiments

In vitro permeation experiments were performed with an Ussing chamber using the ileal membranes pretreated with either PBS (control) or hyaluronidase as described above. Following the 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 (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 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. 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 permeation markers, respectively. It was suggested by several literatures that some of the commercially available FDs supplied from Sigma-Aldrich Co. Ltd. contain free FITC. In fact, in some cases, further purification is required for the study [38]. 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 literatures [38,39]. Therefore, the influence of the FD-4 impurity on the permeation coefficients, if any, can be thought to be 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 μM for insulin, 10 mg/mL for FD-4, and 4 mg/mL for antipyrine. The insulin solution was prepared as the same way of *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 (Isc) were monitored simultaneously every 10 min during the experiment, and the values for Rm were calculated by PD/Isc, 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. Antipyrine concentration was determined by

HPLC. The HPLC system consisted of a Shimadzu SCL-10A system controller, SIL-10A autoinjector, LC-10AS liquid chromatograph, SPD-6A UV spectrophotometric detector and 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 mm x 4.6 mm). The flow rate was 2 mL/min and the ultraviolet/visible detector was set at 254 nm.

The steady-state flux (Jss) was calculated from the linear portion of the cumulative flux-vs-time curve. The apparent permeability coefficient (Papp) of each solute was calculated as shown below:

$$Papp = Jss / C$$

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 Papp for PBS-pretreated (control) group ($Papp_{(PBS)}$) and hyaluronidase-pretreated group ($Papp_{(Hyl)}$), respectively.

2.5. *In situ* absorption experiments with co-administration of aprotinin

In situ absorption experiment using the ileum pretreated by hyaluronidase via "exposure" was carried out as described in Chapter 1. Briefly, 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. 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. 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 4hr 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). The resultant blood glucose levels were described as a percentage of pre-dose glucose level and the extent of hypoglycemic response was calculated as the area above the curve ($[AAC]_G$) for 0-4 hr using the trapezoidal method.

2.6. Statistical analysis

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

3. Results

3.1. Degradation of insulin in the rinse solutions

Figure 9 shows profiles of the degradation of insulin in the rinse solutions collected before or after the pretreatment. 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 higher than that in the solution collected after PBS pretreatment (111 min vs. 387 min for hyaluronidase- vs. PBS-treated group; $p<0.05$). These results suggest that insulin was extensively degraded in the compartment removed by hyaluronidase pretreatment i.e. the mucous/glycocalyx layers compartment. Meanwhile, in the rinse solution collected before the pretreatment, i.e. the luminal fluid, insulin was quickly degraded, even though the solution was diluted 10-fold (14.0 ± 0.1 min). This implies that extensive proteolytic degradation occurred in the intestinal tract in quite a short time.

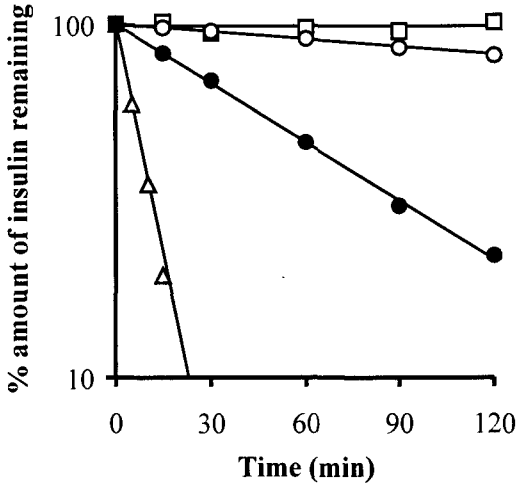

Figure 9. Degradation profile of insulin as a function of time in the rinse solutions.

Keys: (□) PBS solution (control), (○) PBS pretreatment, (●) hyaluronidase pretreatment, and (△) before pretreatment (ten times dilution). Data represent the mean ± SE from n = 3 - 4.

3.2. *In vitro* permeation experiments

Figure 10 and Table 4 show the permeation profiles and the Papp of insulin, FD-4 and antipyrine across the ileal membranes pretreated with PBS and hyaluronidase. The Papp of insulin for the hyaluronidase-treated group was significantly increased compared to the PBS-treated (control) group. On the other hand, the Papp of FD-4 and antipyrine exhibited no significant differences between PBS-pretreated (control) and hyaluronidase- pretreated group. The ratio of Papp (hyaluronidase-treated) / Papp (PBS-treated) values of insulin was four, on the other hand, these of FD-4 and antipyrine were almost one (Table 4).

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. Especially, the percentage ratio of $R_{m/g}$ to R_{total} for insulin were prominent; 74.9 %. On the other hand, the percentage ratio of $R_{m/g}$ to R_{total} for FD-4 and antipyrine were modest; FD-4, -2.3 %; antipyrine, 2.6 %.

Table 4. Papp of insulin, FD-4 and antipyrine during permeation across the rat ileum with or without hyaluronidase pretreatment

	Papp (10^{-8}cm/s)		
	Pretreatment		
Solute	PBS	Hyaluronidase	Ratio
Insulin	0.358 ± 0.051	1.429 ± 0.072*	4.0
FD-4	61.44 ± 3.54	60.04 ± 6.05	1.0
Antipyrine	264.8 ± 27.1	272.0 ± 15.8	1.0

Data: mean ± SE (n = 4-7). * $p < 0.05$ against control. There were no significant differences in the groups of FD-4 and antipyrine.

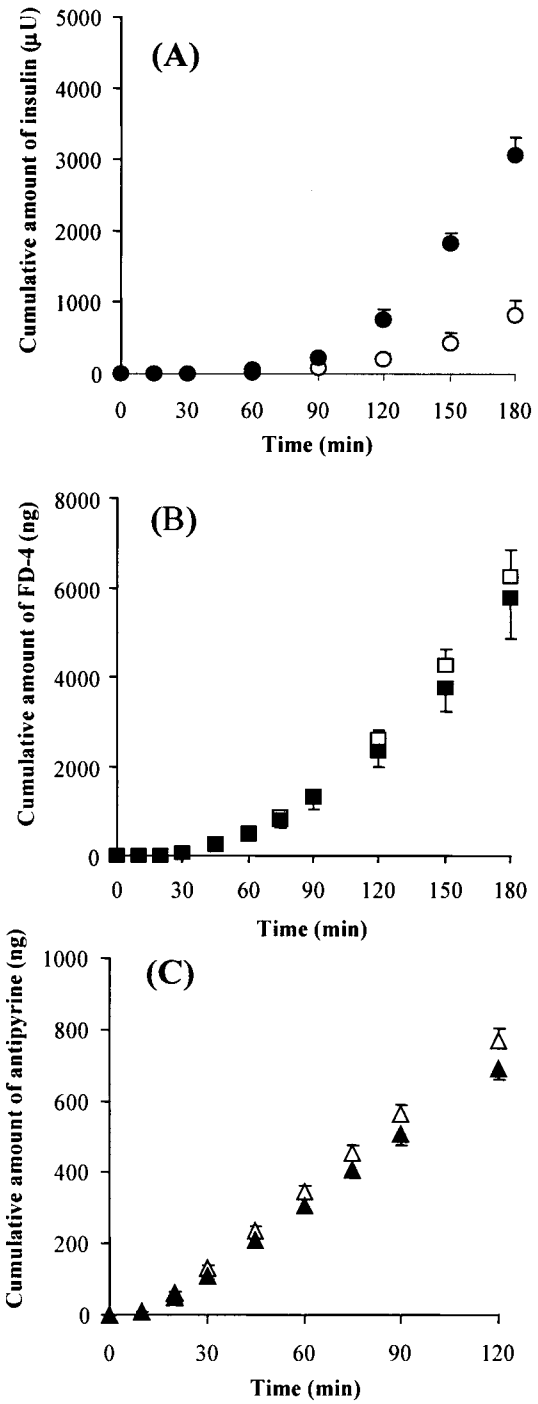

Figure 10. Time course of (A) insulin, (B) FD-4, and (C) antipyrine permeation across rat ileal segments pretreated with PBS and hyaluronidase at 192,000 U/mL.

Keys: PBS (open symbol), hyaluronidase (closed symbol); (○, ●) insulin; (□, ■) FD-4; (△, ▲) antipyrine. Data represent the mean ± SE from n = 4 - 7.

3.3. *In situ* absorption experiments with co-administration of aprotinin

Figure 11 shows the effects of aprotinin on (A) plasma insulin levels and (B) blood glucose levels following 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 5. 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.

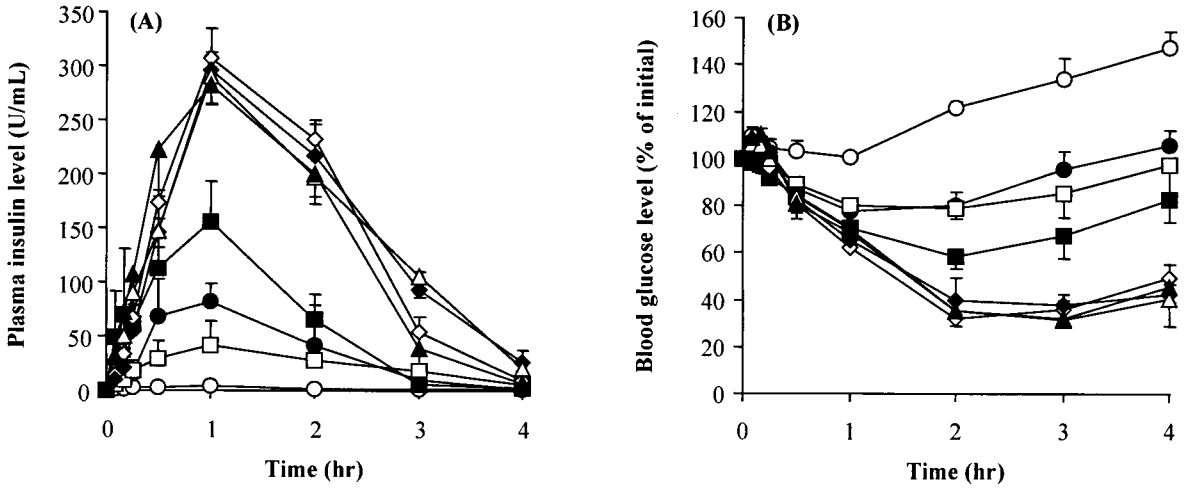

Figure 11. (A) Plasma insulin level and (B) Blood glucose level versus time profiles following *in situ* administration of insulin (50IU/kg) in the presence of aprotinin into the ileal segments pretreated with PBS and hyaluronidase at 192,000 U/mL.

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 ± SE from n = 4.

Table 5. $[AUC]_{insulin}$ and $[AAC]_G$ following the ileal administration of insulin with aprotinin after hyaluronidase pretreatment

Aprotinin dose (TIU/kg)	Pretreatment	$[AUC]_{insulin}$ (μU/mL·hr)		$[AAC]_G$ (% glu. reduc.·hr)	
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 ± SE (n = 4). * $p < 0.05$ against PBS pretreatment.

4. Discussion

In the *in vitro* permeation experiment, the increase in the Papp of insulin following removal of the mucous/glycocalyx layers was observed. The Papp in the PBS-pretreated groups were less than the reported Papp of insulin from rat ileum; $6.82 \pm 1.87 \times 10^{-7}$ cm s^{-1}; and $1.050 \pm 0.206 \times 10^{-6}$ cm s^{-1} [14,15]. The variation in Papp 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 permeation 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 ($\geq$ 70%) revealed the substantial contribution of the mucous/glycocalyx layers to the whole process of permeation of insulin 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 would reside in the mucous/glycocalyx layers [30,40], and it was confirmed that a significant amount of insulin was degraded in the rinse solution collected after the hyaluronidase pretreatment (Fig. 9). Also, hydrophilic macromolecular compounds diffuse through mucous more slowly compared to hydrophilic low molecular weight compounds [37]. However, the Papp 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 since the Papp of antipyrine, a transcellular permeation 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 like 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 attribute to the removal of the enzymatic barrier compartment, i.e. proteolytic activity of pancreatic enzymes enmeshed in the mucous/glycocalyx 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. Meanwhile, although the molecular weight of insulin is similar to that of FD-4, the Papp of insulin in the hyaluronidase-pretreated group was still much 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 [6,41,42]. Since the intestinal mucosal toxicity of aprotinin was minor, as evaluated by the leakage of Evans blue from the systemic circulation [42], 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, the co-administration of aprotinin further enhanced the absorption of insulin from the ileum pretreated with hyaluronidase, as indicated by $[AUC]_{insulin}$ and $[AAC]_G$ (Fig.11 and Table 5), suggesting the

existence of another enzymatic barrier, which was probably unable to be removed by the hyaluronidase pretreatment. During its permeation through the epithelial membrane, insulin could be exposed to enzymes within the mucous/glycocalyx layers as well as the brush-border membrane region, which was unable to be removed by the hyaluronidase pretreatment. The brush-border membrane has various enzymes, of which type and distribution depend on location [43]. While the enzymes that comprise insulin degradation activity were not fully defined, indeed, the brush-border membrane has insulin-degrading activity [12,13]. A few brush-border membrane enzymes (BBME) capable of degrading insulin, such as insulin degrading enzyme (IDE), have been identified [12]. It was reported that IDE is distributed in various tissues and plays an important role in insulin degradation [44]. Although IDE mainly resides in the cytosol, this enzyme also would be present on the brush-border membrane [12,13]. On the other hand, it has been reported that aprotinin was capable of inhibiting IDE activity [45]. Based on these findings, the fact that IDE was inhibited by aprotinin might account for substantial influence of BBME on insulin permeation.

To access the permeation behavior across small intestinal mucosa, 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 [46,47]. 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 this 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

Significant amount of insulin was degraded in the mucous/glycocalyx layers compartments. Also, the *in vitro* permeation of insulin across the ileal tissue following the diminishment of the mucous/glycocalyx layers clearly increased, whereas those of FD-4 and antipyrine did not change. Therefore, the mucous/glycocalyx layers affected to insulin permeation predominantly not as a diffusional but as an enzymatic barrier

Moreover, co-administration of aprotinin, IDE inhibitor, following the diminishment of the mucous/glycocalyx layers increased the absorption of insulin, suggesting the existence of BBME that influence the absorption of insulin.

CHAPTER 3

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

1. Introduction

As discussed in Chapters 1 and 2, the mucous/glycocalyx layers of rat ileum played a significant role in the absorption of insulin. On the other hand, there were some reports that Papp of insulin differs among various intestinal regions [14,15]. Also, Morishita et al, using an *in situ* absorption study with different intestinal loops washed with saline, reported an obvious increase of insulin absorption in the ileum, the lower part of the small intestine, whereas more absorption fraction in upper part was anticipated due to the morphological aspect in which the surface area of intestinal mucosa more extended toward the upper part [6]. These results imply the impedance of the mucous/glycocalyx layers to the absorption of insulin would be varied among different small intestinal regions.

In this chapter, the regional differences in the contribution of the mucous/glycocalyx layers, as a diffusional and/or enzymatic barrier, to the permeation of insulin across small intestine was investigated by the application of hyaluronidase pretreatment extended to the duodenum and the jejunum.

2. Materials and Methods

2.1. Materials

Crystalline human recombinant insulin, FD-4, antipyrine, hyaluronidase, and sodium taurodeoxycholate were the same one in Chapter 1 and 2. All other reagents were of analytical grade and used as received from the suppliers.

2.2. *In situ* hyaluronidase pretreatment

Hyaluronidase pretreatment via "exposure" was carried out as described in Chapter 2. The pretreatment condition, e.g. the concentration of hyaluronidase (192,000 U/mL) and exposure time (30 min), was selected based on the condition afforded maximum hypoglycemic effect by following administration of insulin and the successful diminishment of the mucous/glycocalyx layers. 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 o.d., 2 mm i.d., Saint-Gobain Norton Co., Ltd., Nagano, Japan). The duodenal loop was made at the first portion of the intestine closest to the stomach (c.a. 6 cm length). The next portion, 5 cm away from the ligament of Treitz, was utilized as the jejunal loop (c.a. 10 cm length). The ileal loop was made at the end of the small intestine, just proximal to the ileo-cecal junction (c.a. 10 cm length). Subsequently, these loops were securely ligated to prevent fluid loss. The loops were gently rinsed with 20 mL of PBS 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 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, 2700 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.

2.3. Transmission electron microscopy

The tissue fixation procedure was the same as described in Chapter 1. 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 TEM (H-7000, Hitachi Ltd., Tokyo, Japan) to evaluate the diminishment of the mucous/glycocalyx layers.

2.4. Histological and biochemical examination of the intestinal membranes

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 [22,24]. The experimental methods for LM and LDH leakage were the same as described in Chapter 1.

2.5. *In vitro* permeation experiments

The experimental methods were described in Chapter 2. The concentration in the donor side was adjusted to 200 μM or 1000 μM for insulin, 10 mg/mL for FD-4, and 4 mg/mL for antipyrine, respectively. The values for Rm of used intestinal membrane in steady-state were in the range of 50-90, 40-70 and 30-50 $\Omega \cdot cm^2$ in the duodenum, the jejunum and the ileum, respectively. The values for Rm were greater than 80% of the start value at the end of each test period and the course of Rm during the test period showed no difference between PBS-

and hyaluronidase-treated groups, supporting that the viability of the intestinal membrane was maintained.

2.6. Degradation of insulin in rinse solutions

The experimental methods were described in Chapter 2. HPLC analysis was performed with a Waters Alliances HPLC system (Nihon Waters K.K., Tokyo, Japan)

2.7. Statistical analysis

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

3. Results

3.1. Mucous/glycocalyx layers following hyaluronidase pretreatment

Figures 12 and 13 show light micrographs (AB stain) and electron micrographs of the PBS-treated and the hyaluronidase-treated intestinal mucosal membranes in the duodenum and the jejunum. From light microscopic observations (AB stain), intense staining along the apical side of the epithelial membranes was clearly observed for the PBS-treated (control) group irrespective of intestinal region, indicating the existence of the glycocalyx. In contrast, such a layer was hardly observed for the hyaluronidase-treated group irrespective of intestinal region; 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 70-100 nm thickness of the

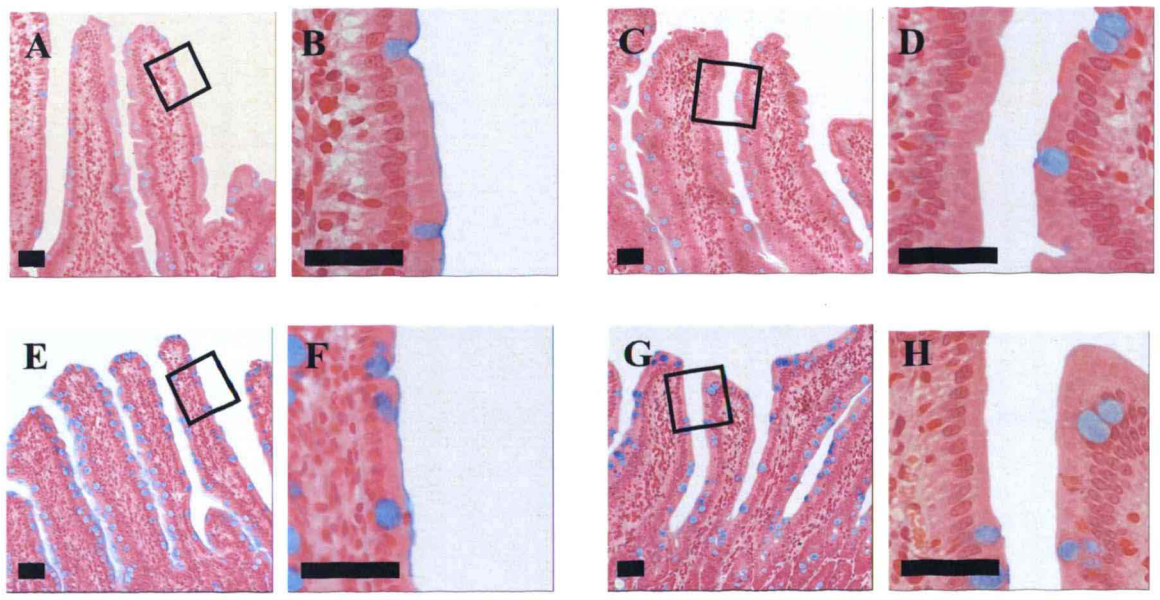

Figure 12. Light micrographs of small intestinal mucosa pretreated with PBS (A, B, E and F) and hyaluronidase at 192,000 U/mL (C, D, G and H): duodenum (A and C; x 10, B and D; x 40), jejunum (E and G; x 10, F and H; x 40).

The scale bar (thick bar) was 40 μm. Tissues were stained with AB (pH 2.5) following fixation using paraformaldehyde (4.0 %).

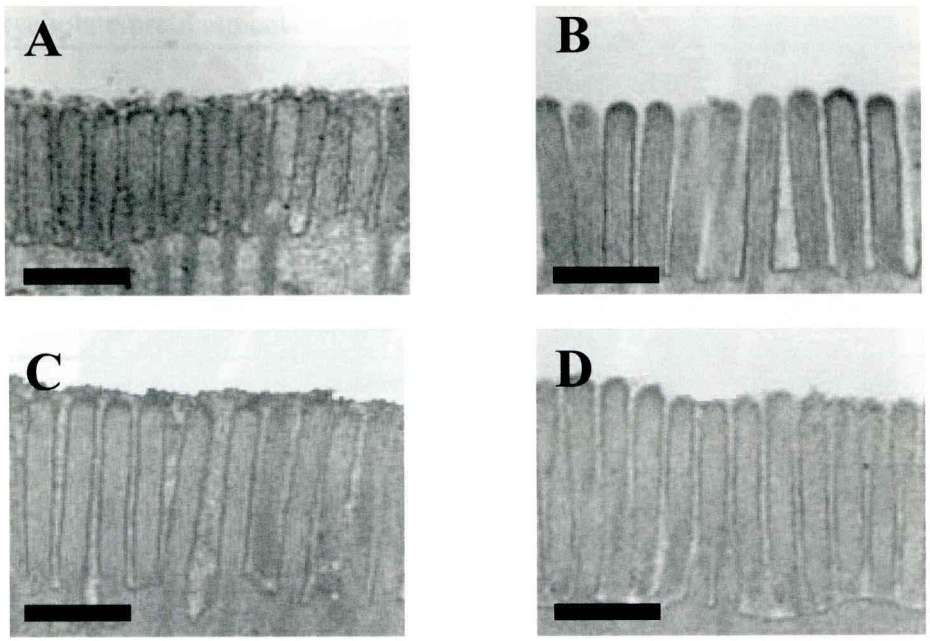

Figure 13. Electron micrographs of small intestinal mucosal membranes pretreated with PBS (A and C) and hyaluronidase at 192,000 U/mL (B and D): duodenum (A and B) and jejunum (C and D).

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 %).

glycocalyx layer was clearly observed as electron-dense layers, enveloping the microvillus consistently for PBS-treated (control) group irrespective of intestinal region. In contrast, the hyaluronidase pretreatment was found to diminish such a layer remarkably, resulting in a near-naked microvillus.

3.2. Histological and biochemical examination of small intestinal membranes

Figure 14 shows light micrographs (HE stain) of the PBS-treated and the hyaluronidase-treated intestinal mucosa in the duodenum and the jejunum. 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.

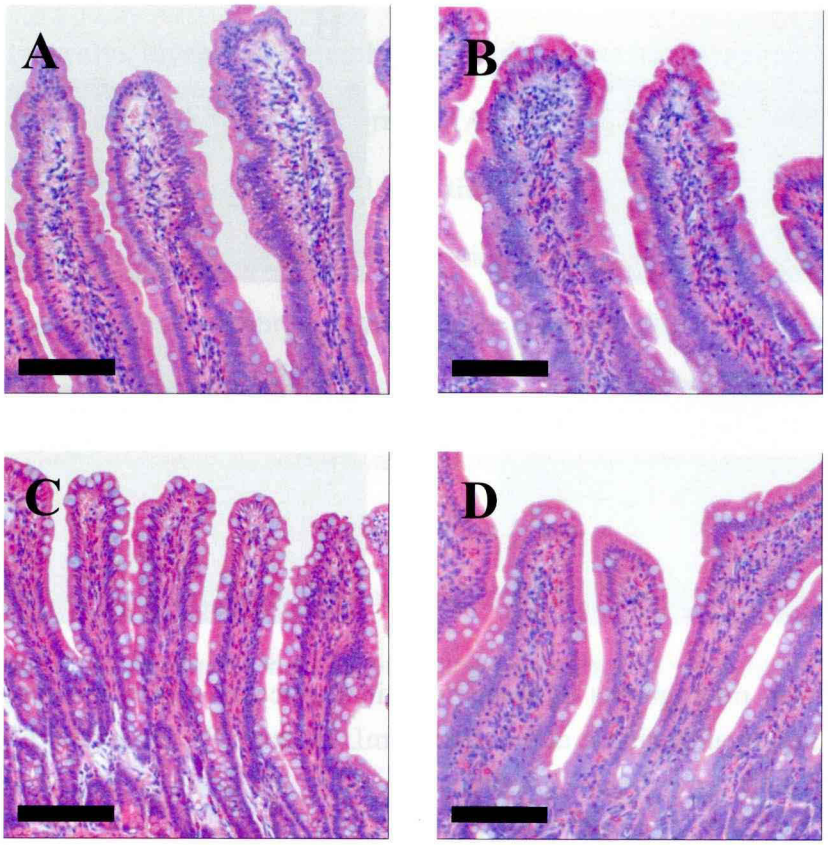

Figure 14. Light micrographs of small intestinal mucosa pretreated with PBS (A and C) and hyaluronidase at 192,000 U/mL (B and D): duodenum (A and B), and jejunum (C and D).

The scale bar (thick bar) was 100 μm. Tissues were stained with HE following fixation using paraformaldehyde (4.0 %).

Table 6 shows the LDH leakage for PBS and hyaluronidase pretreatment irrespective of small intestinal region. Following the hyaluronidase pretreatment, LDH leakage into the mucosal lumen was negligible, which was similar to the leakage seen in the PBS-treated group. In contrast, LDH leakage was dramatically increased with 1.0 % (w/v) sodium taurodeoxycholate using as positive control.

Table 6. 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 Chapter 2. Each value represents the mean with standard error of group of three to four experiments. * $p < 0.05$ against control.

3.3. *In vitro* permeation experiments

Figure 15, Figure 16 and Table 7 show the permeation profiles and the Papp of insulin, FD-4 and antipyrine across the various intestinal membranes pretreated with PBS and hyaluronidase. The Papp 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 1000 μM. Note; at a donor side concentration of 200 μM, only a small amount of insulin appeared on the receiver side. Therefore, the steady-state flux (Jss) could not be calculated, thus, only the Papp at a donor side concentration of 1000 μM were shown in Table 7. The Papp of insulin in both PBS-pretreated (control) and hyaluronidase- groups increased in the following order, duodenum < jejunum < ileum. Values ranged from 0.05 x 10^{-8} to 0.47 x 10^{-8} cm s^{-1} for the PBS-pretreated group and from 0.14 x 10^{-8} to 0.95 x 10^{-8} cm s^{-1} for the hyaluronidase-pretreated group. On the other hand, irrespective of small intestinal region, the Papp of FD-4 and antipyrine exhibited no significant differences between PBS-pretreated (control) and hyaluronidase- pretreated group. The Papp 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 Papp order of

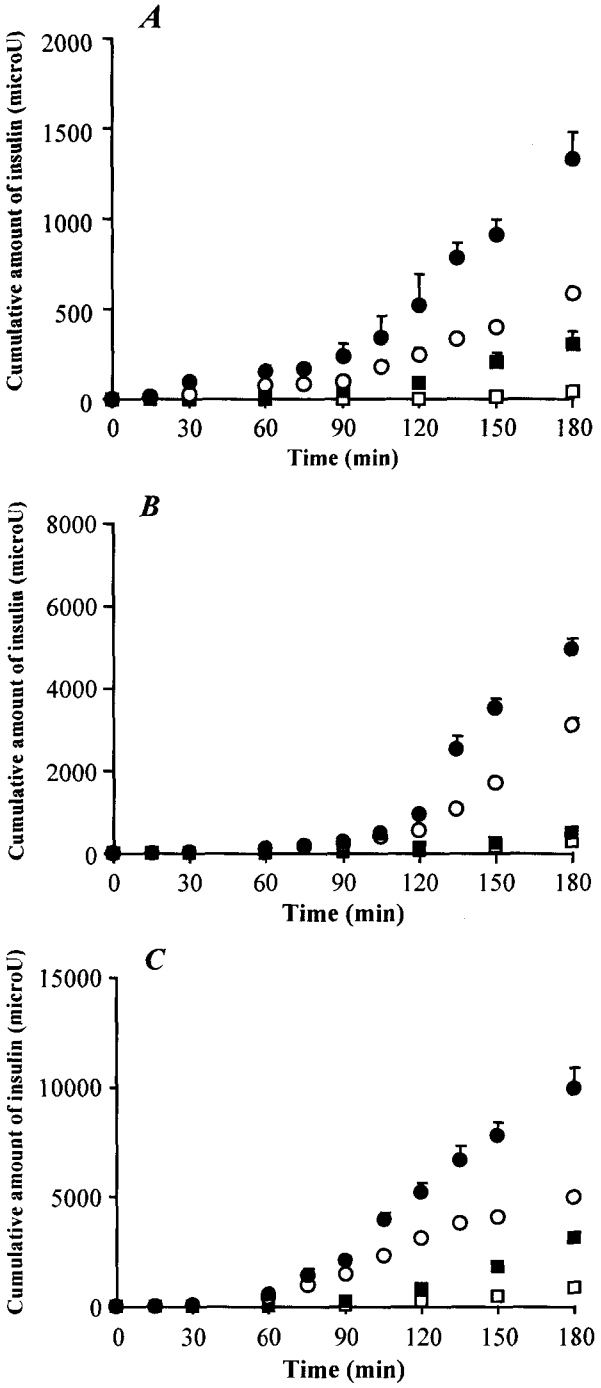

Figure 15. Time course of insulin transport across rat small intestinal segments pretreated with PBS and hyaluronidase at 192,000 U/mL; (A), duodenum; (B), jejunum; (C), ileum.

Keys: PBS (open symbol), hyaluronidase (closed symbol); donor concentration of insulin at 200 μM (□, ■), at 1000 μM (○, ●). Each data represents mean ± SE from n = 4-7.

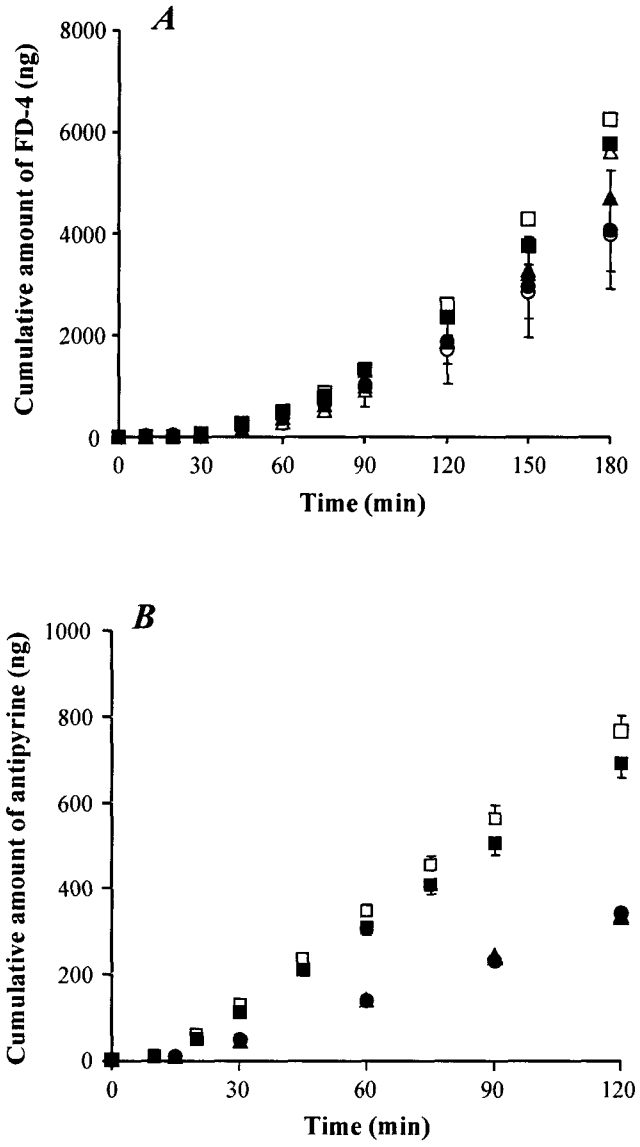

Figure 16. Time course of (A) FD-4 and (B) antipyrine transport across rat small intestinal segments pretreated with PBS and hyaluronidase at 192,000 U/mL.

Keys: PBS (open symbol), hyaluronidase (closed symbol); (○, ●), duodenum; (△, ▲), jejunum; (□, ■), ileum. Each data represents mean ± SE from n = 4-7.

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. Especially, 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; FD-4, 4.4 %, -0.3 % and -2.3 %; antipyrine, 11.5 %, 7.5 % and 2.6 % for the duodenum, the jejunum and the ileum, respectively.

Table 7. Papp of insulin, FD-4 and antipyrine during transport across the various small intestinal membranes with or without hyaluronidase pretreatment

		Papp (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 Chapter 2. Each value represents the mean with standard error of group of four to seven experiments. * $p < 0.05$ against control.

3.4. Degradation of insulin in the rinse solutions

Figure 17 and Table 8 show profiles and half-life of the degradation of insulin in the rinse solutions collected after pretreatment. For all regions, a substantial amount of insulin

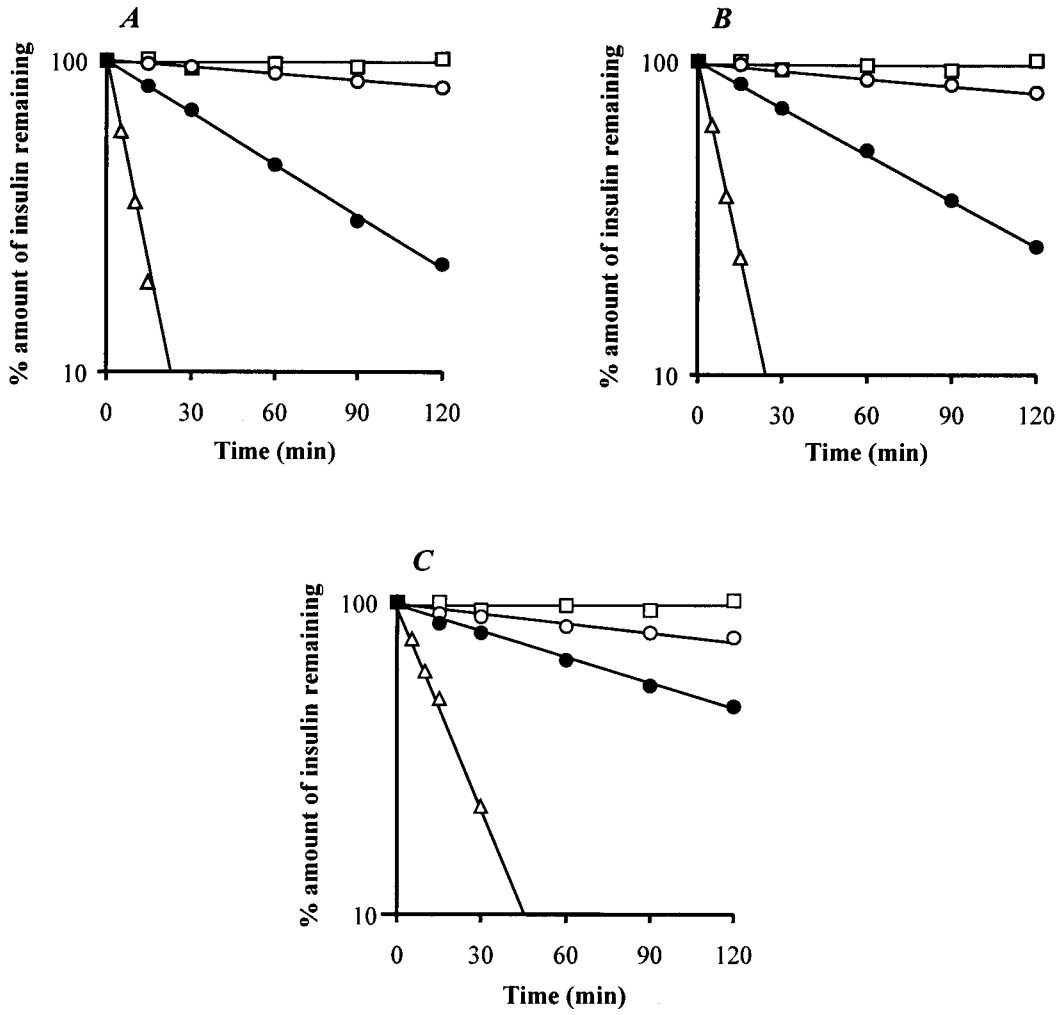

Figure 17. Degradation profile of insulin as a function of time in the rinse solutions: duodenum (A), jejunum (B), ileum (C).

Keys: (□) PBS solution (control), (○) PBS pretreatment, (●) hyaluronidase pretreatment, and (△) before pretreatment (ten times dilution). Each data represents mean ± SE from n = 3-4.

was degraded in the rinse solutions 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 min vs. 428 min, 61 min vs. 400 min and 111 min 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 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, even though the solution was diluted 10-fold (6.5 ± 0.4 min, 7.2 ± 0.2 min 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 quite a short time.

Table 8. The 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 [a)]	PBS	385.8 ± 92.0
	Hyaluronidase	110.5 ± 5.1 *

a) These values are from Chapter 2. Each value represents the mean with standard error of group of four experiments. * $p < 0.05$ against control (PBS pretreatment).

4. Discussion

In Chapter 1, it was demonstrated that the hyaluronidase pretreatment successfully removed the mucous/glycocalyx layers of the rat ileum. In this study, the application of hyaluronidase pretreatment was extended to other parts of the small intestine, and it was demonstrated that the hyaluronidase pretreatment immutably could remove the mucous/glycocalyx layers of each small intestinal segment without damaging membrane integrity as shown by microscopic observations and LDH leakage values (Figs. 12-14, Table 6). Therefore, irrespective of small intestinal region, the hyaluronidase pretreatment technique would allow for precise investigation into the contribution of the mucous/glycocalyx layers to the permeation of certain drugs across the small intestinal mucosa.

In this study, the increase in the Papp of insulin was observed due to the removal of the mucous/glycocalyx layers, irrespective of small intestinal region. These results concur with the results of experiment using the ileal segment in Chapter 2. The Papp in the PBS-pretreated groups were less than the reported Papp 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} [14,15]. The variation in Papp could be explained by differences in apparatus, tissue preparation, concentrations of insulin in donor side studied, analytical method employed and the duration of experiments.

From morphological aspects, there are some differences of the surface area due to the height of intestinal villi and conformation of plica circulares for three separated small intestinal segments, the duodenum, the jejunum and the ileum, whereas the organizations of epithelial cell layer are essentially the same, irrespective of small intestinal region. Therefore, irrespective of small intestinal region, for hydrophilic macromolecules such as insulin and

FD-4, the most likely route of permeation from the mucosal side to the serosal side is the paracellular pathway due to their macromolecular weight.

Considering the permeation resistances of the mucous/glycocalyx layers and the intrinsic intestinal membrane in the paracellular pathway, the prominent percentage ratio of $R_{m/g}$ to R_{total} for insulin ($\geq$ 50%) revealed the substantial contribution of the mucous/glycocalyx layers to the whole process of the permeation of insulin across the intestinal mucosa, irrespective of small intestinal region.

Comparing the results for the duodenum, the jejunum and the ileum, the relationship of Papp of insulin, FD-4, and antipyrine between PBS-treated group (control group) and hyaluronidase- pretreated group are similar among each small intestinal region. Also, significant degradation of insulin occurred in the rinse solution collected after the hyaluronidase pretreatment, irrespective of small intestinal region. Therefore, as well as discussed the results for the ileum in chapter 2, it is clear that the mucous/glycocalyx layers affected the permeation of insulin predominantly as an enzymatic barrier and not as a diffusional barrier, irrespective of small intestinal region.

On the other hand, the Papp 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 of Papp of insulin toward distal small intestinal regions was consistent with those of previous reports of *in vitro* insulin permeation studies using different small intestinal segments [14,15]. Morishita et al, using an *in situ* absorption study with different intestinal loops washed with saline, reported an obvious increase of insulin absorption in the ileum, the distal part of the small intestine [6]. Assuming that the mucous/glycocalyx layers act predominantly as an enzymatic barrier to insulin permeation irrespective of small intestinal region, the order of degradation half-life of insulin in the rinse solution collected after the hyaluronidase pretreatment (duodenum > jejunum > ileum (Table

8)) should correspond to the relative magnitude of enzymatic degradation activity in the mucous/glycocalyx layers in the small intestinal regions. The variation of 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 [48]. The pancreatic enzyme activity of trypsin and chymotrypsin in the duodenum decreases to almost half in the jejunum and to one-third in the ileum [49]. On the other hand, only slight differences of Papp for FD-4 existed among the small intestinal regions (Table 7), which is consistent with a previous report that showed there were no significant differences of the Papp for FD-3 (FITC-dextran, average molecular weight of 3 kDa) between the jejunum and the ileum [50]. From this, it is deduced that there are no significant regional differences in the intrinsic permeation resistance of the intestinal membrane to insulin. Therefore, the variation of the enzymatic activity in the mucous/glycocalyx layers is one of the main factors that account for the regional differences in the permeation of insulin.

Meanwhile, comparing the Papp of insulin and FD-4 in the hyaluronidase-pretreated group, the Papp of insulin in the hyaluronidase-pretreated group was still much lower than that of FD-4, irrespective of small intestinal region, although the molecular weight of insulin is similar to that of FD-4. This indicates the existence of another enzymatic barrier other than that present in the mucous/glycocalyx layers, i.e. brush border membrane region. The order of Papp of insulin in the hyaluronidase-pretreated group, duodenum < jejunum < ileum (Table 7), 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. In the view of practical needs for peroral delivery of enzymatic

labile drugs such as insulin, considering the regional differences in degradation activity in not only the mucous/glycocalyx layers but brush-border membrane would be helpful to design a rational strategy to increase drug absorption toward therapeutically effective level by controlling resistance levels in the these layers.

5. Conclusions

The mucous/glycocalyx layers could immutably be removed without causing detectable damage to the membrane integrity by the hyaluronidase pretreatment, irrespective of small intestinal regions. From the *in vitro* permeation behavior of insulin across small intestinal mucosa and *in vitro* degradation behavior of insulin in the mucous/glycocalyx layers compartments, it was suggested 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. The Papp of insulin in hyaluronidase groups (i.e. without the mucous/glycocalyx layers) were much less than that of FD-4 and showed regional differences in the following order, duodenum < jejunum < ileum. These results indicated the enzymatic barriers to the permeation of insulin in the mucous/glycocalyx layers and also BBME tend to increase toward the upper small intestine in the following order, duodenum > jejunum > ileum, which would be one factor that accounts for the regional differences in the permeation of insulin.

SUMMARY

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

Of these barrier compartments, recent evidence has suggested that the mucous/ glycocalyx layers, extracellular domains directly attached to the intestinal epithelium, could function significantly as barrier to certain amino acids, nutrients and macromolecules, even compared to the intestinal epithelium. Nevertheless, the information regarding these layers and specifically, polypeptidic molecules (i.e., insulin) has been scarce and thus, the layers were not under-exploited to fully understand their roles and functions in these macromolecular behaviors, which should be differentiated from those of other kinetic barrier compartments (i.e., intestinal epithelium).

In this thesis, the role of the mucous/glycocalyx layers to the absorption of insulin from small intestine was elucidated. Substantial barrier property of these layers i.e., diffusional and enzymatic barrier to the permeation of insulin, and the regional differences in these barrier properties were investigated. In addition, the existence of BBME as enzymatic barrier to the absorption of insulin was investigated.

In Chapter 1, the impedance of the mucous/glycocalyx layers to the absorption of insulin from rat ileum following diminishment of the mucous/glycocalyx layers by hyaluronidase pretreatment was evaluated, compared to the absorption of FDs as non-peptide hydrophilic macromolecules. Successful diminishment of the mucous/glycocalyx layers of the ileal

epithelium without causing detectable damage in intra- and inter-cellular integrity was enable by the hyaluronidase pretreatment via both "perfusion" and "exposure". The absorption of insulin was increased dramatically following diminishment of the mucous/glycocalyx layers, whereas those of FDs were unaffected, indicating the mucous/glycocalyx layers impede the absorption of insulin from the ileum.

As coexistent barrier functions, the mucous/glycocalyx layers have been thought to function as diffusional barrier and/or enzymatic barrier to the absorption of insulin. In Chapter 2, the contribution of mucous/glycocalyx layers, as a diffusional or enzymatic barrier, to the absorption of insulin was further investigated. Significant amount of insulin was degraded in the mucous/glycocalyx layers compartments. Also, the *in vitro* permeation of insulin across the ileal mucosal tissue following diminishment of the mucous/glycocalyx layers was significantly increased, whereas these of FD-4 and antipyrine, stable passive para- and transcellular permeation markers, were unchanged. These indicated that the mucous/glycocalyx layers affected to the permeation of insulin predominantly not as a diffusional but as an enzymatic barrier. On the other hand, besides the enzymatic barrier of the mucous/glycocalyx layers, the BBME was thought to function as enzymatic barrier to the absorption of insulin. In Chapter 2, co-administration of aprotinin, the IDE inhibitor, following the diminishment of the mucous/glycocalyx layers increased the absorption of insulin, suggesting the existence of BBME that influences the absorption of insulin.

In Chapter 3, the regional differences of the impedance of the mucous/glycocalyx layers to the permeation of insulin among small intestinal regions were evaluated. The *in vitro* permeation behavior of insulin across small intestinal mucosa and *in vitro* degradation behavior of insulin in the mucous/glycocalyx layers comportments indicated that the mucous/glycocalyx layers contribute to the permeation of insulin predominantly as an enzymatic barrier and not as a diffusional barrier, irrespective of small intestinal regions. The

enzymatic barriers to the permeation of insulin in the mucous/glycocalyx layers and also BBME tend to increase toward the upper small intestine in the following order, duodenum > jejunum > ileum. These results indicated the impedance of the mucous/glycocalyx layers and BBME were much lower in the ileum, the lower part of small intestine.

In conclusion, it was elucidated that the absorption of insulin from small intestinal mucosa was impeded by the mucous/glycocalyx layers as enzymatic barrier but not as diffusional barrier and also impeded by BBME, of which impedance increased toward the upper part of small intestine. This provides promising advance for design a rational strategy to increase the absorption of insulin toward therapeutically effective level by controlling resistance levels in the mucous/glycocalyx layers.

ACKNOWLEDGMENTS

First, I would like to express my gratitude and appreciation from my heart to Professor, Dr. Kozo Takayama and Associate Professor, Dr. Mariko Morishita (Department of Pharmaceutics, Hoshi University) for helpful guidance in my research work and preparing this dissertation.

Secondly, I wish to thank former president of Hoshi University, Dr. Tsuneji Nagai for their valuable assistance in my research work.

Further, I wish to thank Assistant Professor, Dr. Masahiro Sakagami (Department of Pharmaceutics, School of Pharmacy, Virginia Commonwealth University), Mr. Kazunori Asai (Department of Pharmacy, Matsudo City Hospital), Mr. Bunshiro Akikusa, and Mr. Shuji Hosoda (Department of Pathology, Matsudo City Hospital) for their helpful guidance and assistance in my research work.

Also, I wish to thank Mr. Takao Okamura and Mr. Yoshifumi Kaneko (Research Center, Kyorin Pharmaceutical Co., Ltd.) for their helpful assistance in my research work.

Moreover, I would like to thank Professor, Dr. Yoshie Maitani, Dr. Yasuko Obata, Dr. Koji Nakamura, Mr. Yoshinori Onuki, and the member of the Department of Pharmaceutics for their kindness and friendship.

In addition, I would like to thank Ms. Akiko Nagano and Mr. Junpei Ehara for assistant in my research.

Finally, I will be forever in debt to my family for their support, words and comprehension.

REFERENCES

1) Sayani, A. P., and Chien, Y. W., Systemic delivery of peptides and proteins across absorptive mucosae, *Crit. Rev. Ther. Drug Carrier Syst.*, **13**, 85-184 (1986).

2) Pillai, O., Nair, V., Poduri, R., and Panchagnula, R., Transdermal iontophoresis. Part II: Peptide and protein delivery, *Methods Find Exp. Clin. Pharmacol.*, **21**, 229-240 (1999).

3) Madison, L. L., Combes, B., Unger, R. H., and Kaplan, N., The relationship between the mechanism of action of the sulfonylureas and the secretion of insulin into the portal circulation. *Ann. N. Y. Acad. Sci.* **74**:548-556 (1959).

4) Audus, K. L., and Raub, T. J., Biological Barriers to Protein Delivery, Plenum Press, New York, 1993.

5) Lee, V. H. L., Marcel Dekker, New York, 1991.

6) Morishita, M., Morishita, I., Takayama, K., Machida, Y., and Nagai, T., Site-dependent effect of aprotinin, sodium caprate, Na_2EDTA and sodium glycocholate on intestinal absorption of insulin, *Biol. Pharm. Bull.*, **16**, 68-72 (1993).

7) Ziv, E., and Bendayan, M., Intestinal absorption of peptides through the enterocytes, *Microsc. Res. Tech.*, **49**, 346-352 (2000).

8) Yamamoto, A., Taniguchi, T., Rikyuu, K., Tsuji, T., Fujita, T., Murakami, M., and Muranishi, S., Effects of various protease inhibitors on the intestinal absorption and degradation of insulin in rats, *Pharm. Res.*, **11**, 1496-1500 (1994).

9) Watts, T. L., and Fasano, A., Modulation of intestinal permeability: a novel and innovative approach for the oral delivery of drugs, macromolecules and antigens, *Biotechnol. Genet. Eng. Rev.*, **17**, 433-453 (2000).

10) Pappenheimer, J. R., Role of pre-epithelial "unstirred" layers in absorption of nutrients from the human jejunum, *J. Membrane Biol.*, **179**, 185-204 (2001).

11) Frey, K. T. Giannasca, R. Weltzin, P. J. Giannasca, H. Reggio, W. I. Lencer and M. R. Neutra., Role of the glycocalyx in regulating access of microparticles to apical plasma membranes of intestinal epithelial cells: implications for microbial attachment and oral vaccine targeting, *J. Exp. Med.*, **184**, 1045-1059 (1996).

12) Chang, L. L., and Bai, J. P. F., Evidence for the existence of insulin-degrading enzyme on the brush-border membranes of rat enterocytes, *Pharm. Res.*, **13**, 01-803 (1996).

13) Chang, L. L., Stout, L. E., Wong, W. D., Buls, J. G., Rothenberger, D. A., Shier, W. T., Sorenson, R. L., and Bai, J. P., Immunohistochemical localization of insulin-degrading enzyme along the rat intestine, in the human colon adenocarcinoma cell line (Caco-2), and in human ileum, *J. Pharm. Sci.*, **86**, 116-119 (1997).

14) Asada, H., Douen, T., Waki, M., Adachi, S., Fujita, T., Yamamoto, A., and Muranishi, S., Absorption characteristics of chemically modified-insulin derivatives with various fatty acids in the small and large intestine, *J. Pharm. Sci.*, **84**, 682-687 (1995).

15) Schilling, R. J., and Mitra, A. K., Intestinal mucosal transport of insulin, *Int. J. Pharm.*, **62**, 53-64 (1990).

16) Ito, S., Structure and function of the glycocalyx, *Fed. Proc.*, **28**, 12-25 (1969).

17) Varki, A., Sialic acids as ligands in recognition phenomena, *FASEB J.*, **11**, 248-255 (1997).

18) Wang, G., Williams, G., Xia, H., Hickey, M., Shao, J., Davidson, B. L., and McCray, P. B., Apical barriers to airway epithelial cell gene transfer with amphotropic retroviral vectors, *Gene Ther.*, **14**, 922-931 (2002).

19) Kovbasnjuk, O. N., and Spring, K. R., The apical membrane glycocalyx of MDCK cells, *J. Membrane Biol.*, **176**, 19-29 (2000).

20) Henry, C. B. S., and Duling, B. R., Permeation of the luminal capillary glycocalyx is determined by hyaluronan, *Am. J. Physiol. Heart Circ. Physiol.*, **277**, H508-H514 (1999).

21) Huxley, V. H., and Williams, D. A., Role of a glycocalyx on coronary arteriole permeability to proteins: evidence from enzyme treatments, *Am. J. Physiol. Heart Circ. Physiol.*, **278**, H1177-H1185 (2000).

22) Ennis, R. D., Borden, L., and Lee, W. A., The effects of permeation enhancers on the surface morphology of the rat nasal mucosa: a scanning electron microscopy study, *Pharm. Res.*, **7**, 468-475 (1990).

23) Ito, S., The enteric surface coat on cat intestinal microvilli, *J. Cell Biol.*, **27**, 475-491 (1965).

24) Swenson, E. S., Milisen, W. B., and Curatolo, W., Intestinal permeability enhancement: efficacy, acute local toxicity, and reversibility, *Pharm. Res.*, **11**, 1132-1142 (1994).

25) Bendayan, M., Ziv, E., Gingras, D., Ben-Sasson, R., Bar-On, H., and Kidron, M., Biochemical and morpho-cytochemical evidence for the intestinal absorption of insulin in control and diabetic rats. Comparison between the effectiveness of duodenal and colon mucosa, *Diabetologia*, **37**, 119-126 (1994).

26) Bergeron, J. J. M., Rachubinski, R., Searle, N., Borts, D., Sikstrom, R., and Posner, B. I., Polypeptide hormone receptors in vivo, *J. Histochem. Cytochem.*, **28**, 824-835 (1980).

27) Forgue-Lafitte, M. E., Marescot, M. R., Chamblier, M. C., and Rosselin, G., Evidence for the presence of insulin binding sites in isolated rat intestinal epithelial cells, *Diabetologia*, **19**, 373-378 (1980).

28) Fernandez-Moreno, M. D., Serrano-Rios, M., and Prieto, J. C., Identification of insulin receptors in epithelial cells from duodenum, jejunum, ileum, caecum, colon and rectum in the rat, *Diabete Metab.*, **13**, 135-139 (1987).

29) Woodley, J. F., and Sterchi, E. E., Endopeptidase activity of the small intestine, In: Hemmings, W. A. (ed.), MTP Press Ltd., London, pp.199-205.

30) Ugolev, A. M., Smirnova, L. F., Iezuitova, N. N., Timofeeva, N. M., Mityushova, N. M., Egorova, V. V., and Parshkov, E. M., Distribution of some adsorbed and intrinsic enzymes between the mucosal cells of the rat small intestine and the apical glycocalyx separated from them, *FEBS Lett.*, **104**, 35-38 (1979).

31) Quan, Y. S., Fujita, T., Tohara, D., Tsuji, M., Kohyama, M., and Yamamoto, A., Transport kinetics of leucine enkephalin across Caco-2 monolayers: quantitative analysis for contribution of enzymatic and transport barrier, *Life Sci.*, **64**, 1243-1252 (1999).

32) Forbes, B., Wilson, C. G., and Gumbleton, M., Temporal dependence of ectopeptidase expression in alveolar epithelial cell culture: implications for study of peptide absorption, *Int. J. Pharm.*, **180**, 225-234 (1999).

33) Liu, F. Y., Kildsig, D. O., and Mitra, A. K., Pulmonary biotransformation of insulin in rat and rabbit, *Life Sci.*, **51**, 1683-1689 (1992).

34) Bai, J. P. F., Subcellular distribution of proteolytic activities degrading bioactive peptides and analogues in the rat small intestinal and colonic enterocytes, *J. Pharm. Pharmacol.*, **8**, 671-675 (1994).

35) Ugolev, A. M., and Laey, P. D., Membrane digestion. A concept of enzymic hydrolysis on cell membranes, *Biochim. Biophys. Acta*, **300**, 105-128 (1973).

36) Rubinstein, A., and Tirosh, B., Mucus gel thickness and turnover in the gastrointestinal tract of the rat: response to cholinergic stimulus and implication for mucoadhesion, *Pharm. Res.*, **11**, 794-799 (1994).

37) Desai, M. A., Mutlu, M., and Vadgama, P., A study of macromolecular diffusion through native porcine mucous, *Experientia*, **48**, 22-26 (1992).

38) Preston, R. A., Murphy, R. F., and Jones, E. W., Apparent endocytosis of fluorescein isothiocyanate-conjugated dextran by Saccharomyces cerevisiae reflects uptake of low molecular weight impurities, not dextran, *J. Cell Biol.*, **105**, 1981-1987 (1987).

39) Tomita, M., Menconi, M. J., Delude, R. L., and Fink, M. P., Polarized transport of hydrophilic compounds across rat colonic mucosa from serosa to mucosa is temperature dependent, *Gastroenterology*, **118**, 535-543 (2000).

40) Goldberg, D. M., Campbell, R., and Roy, A. D., Binding of trypsin and chymotrypsin by human intestinal mucosa, *Biochim. Biophys. Acta.*, **167**, 613-615 (1968).

41) Bernkop-Schnurch, A., The use of inhibitory agents to overcome the enzymatic barrier to perorally administered therapeutic peptides and proteins, *J. Control. Release.*, **52**, 1-16 (1998).

42) Uchiyama, T., Sugiyama, T., Quan, Y. S., Kotani, A., Okada, N., Fujita, T., Muranishi, S., and Yamamoto, A., Enhanced permeability of insulin across the rat intestinal membrane by various absorption enhancers: their intestinal mucosal toxicity and absorption-enhancing mechanism of n-lauryl-beta-D-maltopyranoside, *J. Pharm. Pharmacol.*, **51**, 1241-1250 (1999).

43) Bai, J. P., Comparison of distribution of brush-border exo- and endopeptidases in rat and rabbit intestine, *J. Pharm. Pharmacol.*, **46**, 928-930 (1994).

44) Kuo, W. L., Montang, A. G., and Rosner, M. R., Insulin-degrading enzyme is differentially expressed and developmentally regulated in various rat tissues, *Endocrinology*, **132**, 604-611 (1993).

45) Roth, R. A., Mesirow, M. L., Cassell, D. J., Yokono, K., and Baba, S., Characterization of an insulin degrading enzyme from cultured human lymphocytes, *Diabetes Res. Clin. Pract.*, **1**, 31-39 (1985).

46) Conradi, R. A., Hilgers, A. R., Ho, N. F. H., and Burton, P. S., The influence of peptide structure on transport across Caco-2 cells. *Pharm. Res.* **8**, 1453-1460 (1991).

47) Artursson, P., and Karlsson, J., Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells, *Biochem. Biophys. Res. Commun.*, **175**, 880-885 (1991).

48) Layer, P., Jansen, J. B., Cherian, L., Lamers, C. B., and Goebell, H., Feedback regulation of human pancreatic secretion. Effects of protease inhibition on duodenal delivery and small intestinal transit of pancreatic enzymes, *Gastroenterology*, **198**, 1311-1319 (1990).

49) Holtmann, G., Kelly, D. G., Sternby, B., and DiMagno, E. P., Survial of human pancreatic enzymes during small bowel transit: effect of nutrients, bile acids, and enzymes. *Am. J. Physiol.* **273**: G553- 558 (1997).

50) Pantzar, N., Westrom, B. R., Luts, A., and Lundin, S., Regional small-intestinal permeability in vitro to different-sizes dextrans and proteins in the rat, *Scand. J. Gastroenterol.*, **28**, 205-211 (1993).