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

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Purpose. To test the hypothesis that the ileal mucous/glycocalyx layers can be removed by hyaluronidase and that their significant roles in insulin absorption can thereby be identified.

Methods. Rat ileal segments were pretreated with various concentrations of hyaluronidase by "perfusion" or "exposure", and the absorption of insulin and 4.4-, 20-, and 40-kDa fluorescein isothiocyanate-labeled dextrans (FDs) were studied in the *in situ* ileal loop system. Diminished mucous/glycocalyx layers following the hyaluronidase pretreatment was confirmed by transmission electron microscopy (TEM), whereas intra- and intercellular integrity and/or damage was examined by light microscopy, lactate dehydrogenase (LDH) leakage, and membrane electrical resistance (R_m).

Results. Hyaluronidase "perfusion" pretreatment at concentrations $\geq$ 160 U/ml for 30 min significantly increased the hypoglycemic responses following *in situ* administration of insulin at 50 IU/kg. This enhancing effect was found to be dependent on hyaluronidase concentration and "exposure" periods and accompanied by the TEM observation of diminished mucous/glycocalyx layers from the hyaluronidase pretreatment, yet causing undetectable histological damage. In contrast, the absorption of FDs and the values for LDH leakage and R_m were unaffected by the hyaluronidase pretreatment, suggesting that the layers functioned insignificantly to diffusive absorption.

Conclusions. Hyaluronidase pretreatment increased ileal absorption of insulin, but not FDs, by virtue of the diminished mucous/glycocalyx layers without causing detectable cellular damage.

KEY WORDS: insulin; hyaluronidase; mucous; glycocalyx; macromolecular drugs.

INTRODUCTION

Successful oral delivery of macromolecules such as proteins and polypeptides is precluded primarily by insufficient membrane permeability and extensive proteolytic degradation in the intestinal tract (1,2). Accordingly, several *in vivo* and *in vitro* studies using absorption enhancers and enzyme inhibitors have shown enhanced macromolecular absorption (1–6), although 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. Meanwhile, recent evidence has suggested that the mucous and glycocalyx layers, extracellular domains directly attached to the intestinal epithelium, could function significantly as absorptive and/or proteolytic impedimental compartments for certain amino acids, nutrients, and macromolecules, even compared to the intestinal epithelium (7,8). Nevertheless, information regarding these layers and specifically polypeptidic molecules (i.e., insulin) is scarce, and thus, the layers are underexploited to fully understand their roles and functions in these macromolecular behavior, which should be differentiated from those of other kinetic barrier compartments (i.e., intestinal epithelium).

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 (8,9). The layers are believed to protect the apical cell surface against microbial pathogens and foreign materials partially by virtue of electrical repulsion for negatively charged sugar moieties (7,10). 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 (7,10,11). 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, and macromolecules (2,7–9,12–18). Meanwhile, these proteolytic enzymes are also known to be floated in the intestinal fluid, anchored with the epithelium, and localized and functioning in the intestinal cells (13,14,18), and thus, the importance of these glycocalyx enzymes remains uncertain.

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 (11,16,19,20). Notably, these were identified in the experimental systems where enzymes such as hyaluronidase and/or neuraminidase were used in pretreatment to diminish the glycocalyx layers (11,16,19,20). Thus, in this study, we hypothesized that the intestinal (ileal) mucous/glycocalyx layers could be similarly diminished by hyaluronidase pretreatment, and thereby, their significant roles in macromolecular absorption and proteolysis could be identified. Insulin and differently sized, fluorescein isothiocyanate-labeled dextrans (FD-4, FD-20, and FD-40) were used as model macromolecular solutes, and their ileal absorption was studied in the *in situ* rat system following hyaluronidase pretreatment. The diminished mucous/glycocalyx layers following hyaluronidase pretreatment was ensured by transmission electron microscopy (TEM), whereas intra- and intercellular integrity and/or damage were examined by light microscopy, lactate dehydrogenase (LDH) leakage and membrane electrical resistance (R_m).

MATERIALS AND METHODS

Materials

Model macromolecular solutes—crystalline bovine insulin (USP; 28.2 IU/mg), and fluorescein isothiocyanate-labeled

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dextrans (FDs) with weight-averaged molecular weights (MW) of 4.4, 20 and 40 kDa (FD-4, FD-20 and FD-40, respectively)—were obtained from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Lyophilized hyaluronidase (EC 3.2.1.35; Type IV-S from bovine testes; MW = 56 kDa, 1320 U/mg solid) and sodium taurodeoxycholate were also purchased from Sigma. All other reagents were of analytic grade and used as received from the suppliers.

Hyaluronidase Pretreatment and *In Situ* Absorption Experiment

This research was performed at Hoshi University and complied with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals. Male Wistar rats weighing 180–220 g were purchased from Sankyo Lab Service Co., Ltd. (Tokyo, Japan). 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, but they were fasted for 24 h before 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 a 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 tubing (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 pretreatment was given to the ileal segments via "perfusion" to efficiently diminish the mucous/glycocalyx layers. Therefore, as its methodologic validation, transmission electron microscopy (TEM) was used to ensure their diminution, and histologic, biochemical, and electrophysiologic examinations were to evaluate possible membrane damage from 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 was composed of 137 mM NaCl, 2.6 mM KCl, 6.4 mM $Na_2HPO_4 \cdot 12H_2O$, and 1.4 mM KH_2PO_4. 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 h to recover from the elevated blood glucose level caused by the surgical operation described above.

In the *in situ* absorption experiments, 0.5 ml of insulin or each of the FD 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 solution's pH was simultaneously adjusted to 7.4 using 0.1 M NaOH. Each of FD 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 before 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 h 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 percentage of predose glucose level; hence, as referenced with the corresponding blood glucose levels seen in the control (PBS-treated) group, the extent of hypoglycemic response was calculated as the area above the curve ($[AAC]_G$) for 0–4 h using the trapezoidal method. In contrast, each FD's absorption 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 13,400 *g* for 2 min at room temperature. Whereas 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 h 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 insulin's ileal absorption by hyaluronidase administered in a way that is realistically applicable to the situation *in vivo*. The ileal segments were exposed to 1.0 ml (37°C) of PBS (pH 7.4; control) or hyaluronidase in PBS at 9,600, 19,200, 48,000, 96,000, 192,000, and 384,000 U/ml for 30 min, followed by tight closures at their both ends; no perfusion was used. In some experiments, the exposure periods were also varied to 15, 30, 60, and/or 90 min. 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 studies at a dose of 50 IU/kg were performed, as described earlier.

Transmission Electron Microscopy

The ileal segments were pretreated with PBS (control) or 320 U/ml of hyaluronidase in PBS via "perfusion" for 30 min, as described above. The segments were removed from the body and fixed with 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). A secondary fixation used 1.0% osmium tetroxide in the same buffer for 1 h, followed by dehydration and embedding in Epon 812 (NISSIN-EM Co., Ltd., Tokyo, Japan). Thin cross-sectional samples were prepared by ultramicrotome (MT-5000, Du Pont Co., Ltd., Wilmington, DE, USA) and were finally stained with uranyl acetate and lead citrate to be examined by TEM (H-7500, Hitachi Co., Ltd., Tokyo, Japan) for evaluation of the diminution of the mucous/glycocalyx layers.

Histologic, Biochemical, and Electrophysiologic Examination of the Ileal Membranes Following Hyaluronidase Pretreatment

The ileal segments were pretreated with PBS (control) or 320 U/ml of hyaluronidase in PBS via "perfusion" for 30 min, as described above. Some experiments used pretreatment

with 1.0% (w/v) sodium taurodeoxycholate as an active control because of its known induction of mucosal damage (21).

Light Microscopy

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

Lactate Dehydrogenase (LDH) Leakage

Following the "perfusion" pretreatment, the perfused solutions were collected to determine the leakage of LDH, an intracellular enzyme often used to evaluate intracellular integrity (22). 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 (R_m)

Because membrane electrophysiologic parameters such as R_m are not possible to be determined directly in the *in situ* system described above, these were alternatively studied *in vitro* with an Ussing chamber where the ileal membranes pretreated with either PBS or 320 U/mL hyaluronidase in the *in situ* system were mounted. Following "perfusion" 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 was composed of 108.0 mM NaCl, 11.5 mM D-glucose, 15.0 mM $NaHCO_3$, 4.7 mM KCl, 1.8 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 4.9 mM Na-glutamate, 5.4 mM Na_2-fumarate, and 4.9 mM Na-pyruvate. Subsequently, their muscle layer was stripped, 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 before monitoring of the electrophysiologic parameters. The spontaneous transmucosal potential difference (PD) and the short-circuit current (I_{sc}) were recorded simultaneously at every 10 min for 90 min, and the values for R_m were calculated by PD/I_{sc}, based on the Ohm's law. These were corrected by eliminating the offset voltage between the electrodes and series fluid resistance, which was determined before each experiment using the identical bathing solutions, yet in the absence of ileal membranes mounted in the chamber.

Statistical analysis

Each value was expressed as mean ± standard error (SE). Multiple ANOVA was performed, followed by the 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.

RESULTS

Insulin and FD Absorption from the Ileal Segments Pretreated by Hyaluronidase via "Perfusion"

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 did not affect 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 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·h, respectively). Meanwhile, insulin's absorption appeared to be terminated at ≤ 3 h (Fig. 1), implying substantial insulin degradation in the ileal loop and/or relatively short periods of the hyaluronidase effects via "perfusion" on this increased insulin absorption.

Table I 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 insulin's case (Fig. 1), 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 I were not significantly different between the PBS-treated (control) and the hyaluronidase-treated groups.

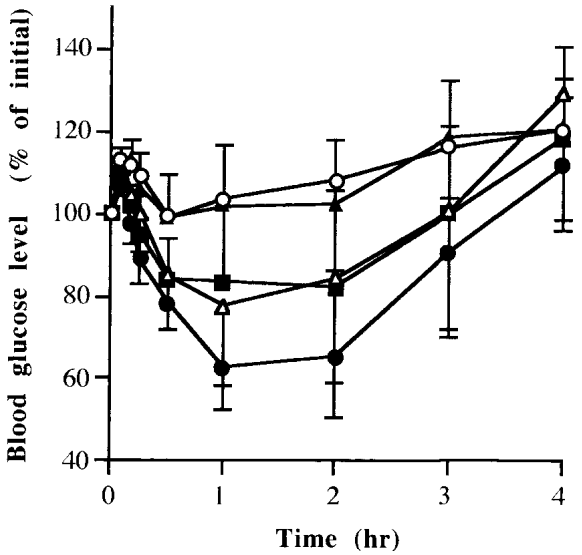

Fig. 1. Blood glucose level vs. time profiles following *in situ* administration of insulin (50 IU/kg) into the ileal segments pretreated with PBS and various concentrations of hyaluronidase for 30 min via "perfusion." Each data represents mean ± SE from n = 4. Key: (○) PBS (control); (▲, △, ●, and ■) hyaluronidase at 80, 160, 320, and 480 U/ml, respectively.

Table I. Pharmacokinetic Parameters Derived from the Plasma Concentration vs. Time Profiles for Fluorescein Isothiocyanate-Labeled Dextrans, FD-4, FD-20, and FD-40, Following *In Situ* Administration into the Ileal Segments Pretreated with PBS (Control) and 320 U/ml Hyaluronidase for 30 min via "Perfusion"

Solute	Pretreatment	C_{max} (μg/ml)	T_{max} (h)	$[AUC]_{FD}$ (μg/ml · h)
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.

This suggested that the hyaluronidase pretreatment did not alter diffusive transport of these macromolecules across the ileal membranes.

Ileal Mucous/Glycocalyx Layers Following Hyaluronidase Pretreatment: TEM

Figure 2 shows electron micrographs of (a) the PBS-treated and (b) the hyaluronidase (320 U/mL)-treated ileal mucosal membranes. Despite the "perfusion" treatment with PBS for 30 min, approximately 70–100 nm thickness of the glycocalyx layer was clearly observed as electron-dense layers, enveloping the ileal microvillus consistently (Fig. 2a); the mucous layer was removed via chemical fixation and dehydration of the ileal segment samples and thus was not observed in any specimens. In contrast, the hyaluronidase pretreatment was found to diminish such a layer remarkably, resulting in the near-naked ileal microvillus (Fig. 2b). These results verified that *in situ* absorption studies described above examined the effects of the mucous/glycocalyx layers on insulin and dextran absorption across the ileal membranes. It was likely, therefore, that the diminution of the mucous/glycocalyx layers caused, in part, the increased absorption of insulin but left unaltered the diffusive absorption of FDs. Meanwhile, the epithelial cellular integrity seemed to be unaffected by the hyaluronidase treatment.

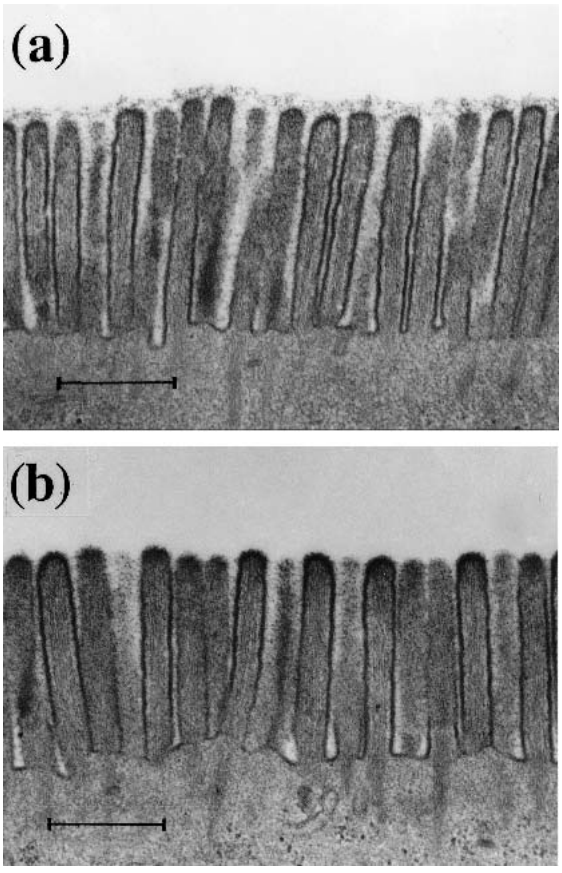

Fig. 2. Electron micrographs of the ileal mucosal membranes pretreated with (a) PBS and (b) 320 U/ml hyaluronidase for 30 min via "perfusion" (×30,000 magnification). The bars indicate 0.5 μm. Tissues were stained with uranyl acetate and lead citrate following primary and secondary fixations using glutaraldehyde (2.5%), paraformaldehyde (2.0%), and osmium tetroxide (1.0%).

Histologic, Biochemical, and Electrophysiologic Examination of the Ileal Membranes Following Hyaluronidase Pretreatment

Histologic micrographs of the ileal mucosal membranes pretreated with (a) PBS, (b) 320 U/ml hyaluronidase, and (c) 1.0% (w/v) sodium taurodeoxycholate for 30 min via "perfusion" are shown in Fig. 3. As observed in the electron micrographs shown in Fig. 2, no apparent histologic damages were found in the hyaluronidase-treated mucosal membranes and cells, compared to those in the PBS-treated (control) counterparts (Fig. 3b and 3a, respectively). This was quite in contrast to the 1.0% (w/v) sodium taurodeoxycholate-treated sections where moderate histological disruptions were clearly observed under the identical protocol, as shown in Fig. 3-c.

Table II shows the LDH leakage and the values for R_m in the absence (PBS; control) and presence of 320 U/ml hyaluronidase "perfusion" pretreatment. LDH was negligibly 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, respectively), whereas 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 R_m were unchanged for 90 min (~25 $\Omega \cdot cm^2$; Table II). These data suggested that the ileal membrane integrity and cellular tight junctions remain mostly unaltered by the hyaluronidase pretreatment used in this study.

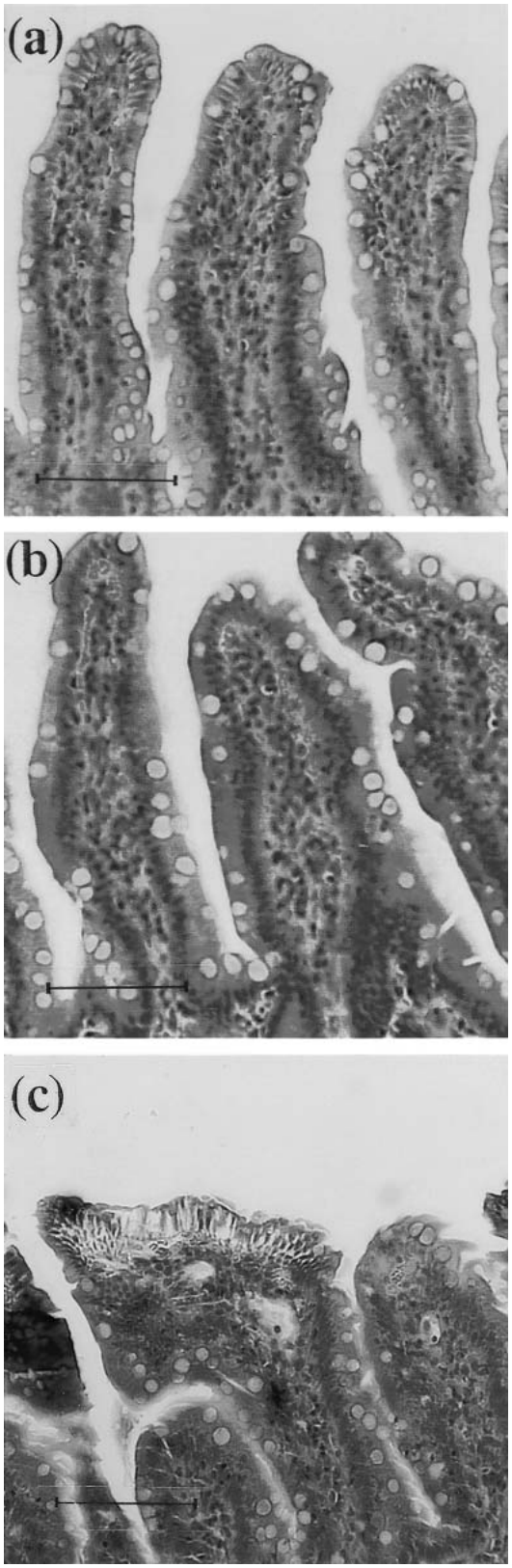

Fig. 3. Light micrographs of the ileal mucosal membranes pretreated with (a) PBS, (b) 320 U/ml hyaluronidase, and (c) 1.0% (w/v) sodium taurodeoxycholate for 30 min via "perfusion" (×200 magnification). The bars indicate 100 μm. Tissues were stained with hematoxylin and eosin following fixation using glutaraldehyde (2.5%) and paraformaldehyde (2.0%).

Table II. Lactate Dehydrogenase (LDH) Leakage and Membrane Electrical Resistance (R_m) Following PBS (Control), 320 U/ml Hyaluronidase, and 1.0% (w/v) Sodium Taurodeoxycholate Pretreatment for 30 min 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 R_m differed insignificantly between the PBS-treated (control) and the hyaluronidase-treated groups over 90 min.

Effects of Hyaluronidase "Exposure" Pretreatment on Insulin Absorption

Although hyaluronidase "perfusion" at 320 U/ml successfully diminished the mucous/glycocalyx layers (Fig. 2) 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 pretreatment was conducted in an "exposure" fashion, mimicking *in vivo* application where hyaluronidase is administered before macromolecular administration. Figure 4 shows the effects of such "exposure" pretreatments at various concentrations of hyaluronidase for 30 min 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 responses were observed via "exposure" at hyaluronidase concentrations over 19,200 U/ml (Fig. 4a). It appeared, furthermore, that the enhancing effects were dose-dependent at concentrations below 192,000 U/ml while reaching the maximum of $[AAC]_G = 87.8 \pm 11.6\%$ glucose reduction·h (Fig. 4b). Meanwhile, as shown in Fig. 5, it was found that this hyaluronidase "exposure" effect of increasing $[AAC]_G$ disappeared when the "exposure" periods were extended to 90 and 60 min for 9,600 and 19,200 U/ml hyaluronidase, respectively. As a result, the maximum enhancing effects were achieved when hyaluronidase was exposed for 60 and 30 min via "exposure," respectively, and hence, it was implied that the enhancing effects were dependent on both concentration and "exposure" periods of hyaluronidase. Because the hyaluronidase pretreatment and the increased insulin absorption were associated with the reduced mucous/glycocalyx layer, this further suggested that some sorts of reversible turnover mechanisms of the mucous/glycocalyx layers could be involved in this observation. Despite the use of much higher hyaluronidase concentrations (9,600–384,000 U/ml), compared to "perfusion," there were no significant changes in LDH leakage between the PBS exposure and the hyaluronidase exposure (1.0 ± 0.6 and 1.2 ± 0.3 U for control and 384,000 U/ml hyaluronidase, respectively).

DISCUSSION

In the present study, we have shown that hyaluronidase pretreatment successfully diminished the mucous/glycocalyx layers of the ileal epithelium (Fig. 2) and thereby induced

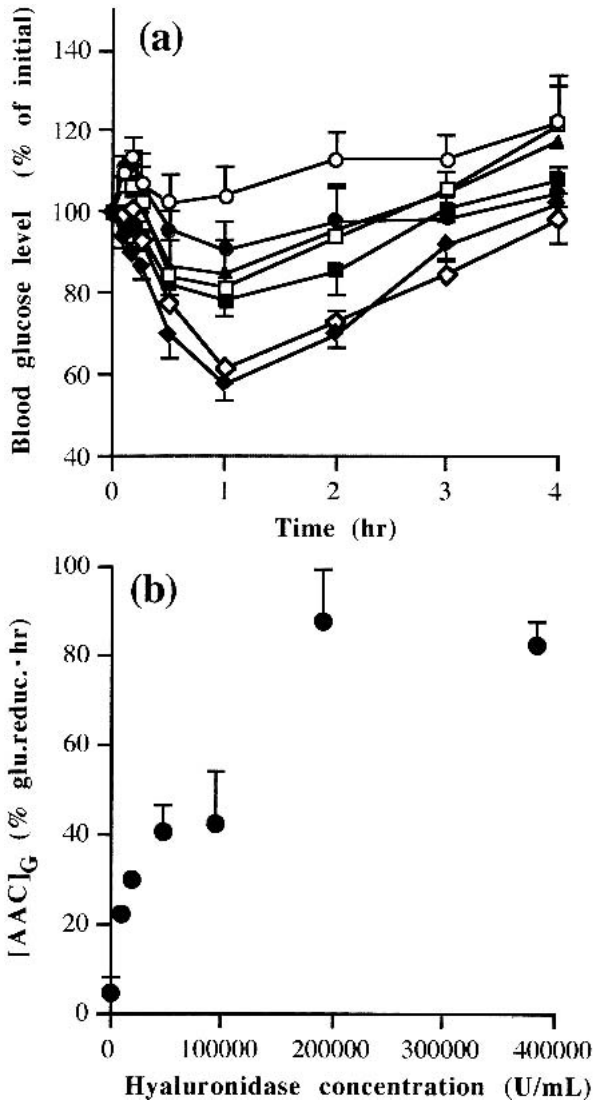

Fig. 4. (a) Blood glucose level vs. time profiles following *in situ* administration of insulin (50 IU/kg) into the ileal segments pretreated with PBS and various concentrations of hyaluronidase for 30 min via "exposure." Key: (○) PBS (control); (●, □, ■, ▲, ◆, and ◇) hyaluronidase at 9,600, 19,200, 48,000, 96,000, 192,000 and 384,000 U/ml, respectively. (b) The calculated $[AAC]_G$ vs. hyaluronidase-pretreated concentration via "exposure." Each data point represents mean ± SE from n = 4.

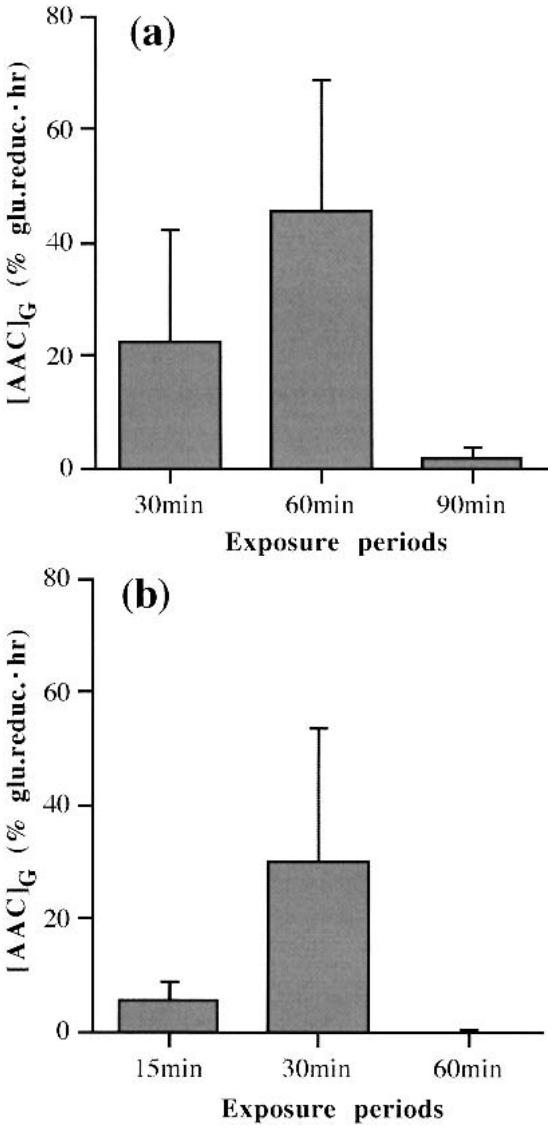

Fig. 5. Effects of "exposure" periods on the values for $[AAC]_G$ following *in situ* administration of insulin (50 IU/kg) into the ileal segments pretreated with (a) 9,600 and (b) 19,200 U/ml of hyaluronidase. Each data point represents mean ± SE from n = 4–6.

significant hypoglycemic responses following insulin administration into the *in situ* ileal lumen (Figs. 1 and 4). In contrast, diffusive absorption of macromolecular dextrans (FD-4, FD-20, and FD-40) was not enhanced following the hyaluronidase pretreatment at a concentration (320 U/ml), causing increased insulin absorption (Fig. 1 and Table I). The microscopic observation alongside the values for LDH leakage and R_m showed that the membrane integrity and the cellular tight junctions appeared to remain undamaged (Fig. 3 and Table II). This is the first demonstration that the ileal epithelial mucous/glycocalyx layers played a significant role specifically in insulin absorption but not in polysaccharide diffusion, and such increased insulin absorption was not caused by cellular damage. Moreover, it has been shown that both "perfusion" and "exposure" pretreatments were effective in increasing insulin absorption, and the magnitudes of the effect appeared to depend on hyaluronidase concentration and "exposure" periods (Figs. 1, 4, and 5).

Unaltered FD absorption (Table I) reasonably implies that the ileal mucous/glycocalyx layers existed as an insignificant impediment in diffusive absorption of macromolecules over 4.4 kDa across the ileal membranes. This was presumably because of much lower intrinsic epithelial membrane permeability for this size of macromolecules, compared to that for diffusion, through the mucous/glycocalyx layers. Unchanged values for LDH leakage and R_m (Table II) following hyaluronidase pretreatment lend strong support to these FD results. Thus, it must be clear that the mechanisms of the increased insulin absorption (Figs. 1, 4, and 5) were not associated with facilitation of intra- and intercellular diffusion. Meanwhile, several authors have speculated on insulin's active absorption through receptor-mediated mechanisms (4,23,24), and in such cases, the increased insulin absorption

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 (7–9). Nevertheless, this possibility is unlikely in view of the extremely high insulin concentration (20 IU/ml) administered in the ileal segments, as opposed to relatively low K_m values generally seen in the literature (25,26).

Another possibility for the increased insulin absorption shown in Figs. 1 and 4 is associated with reduced proteolytic activity by virtue of the diminished mucous/glycocalyx layers following hyaluronidase pretreatment. The hyaluronidase pretreatment diminished the mucous/glycocalyx layers (Fig. 2) and increased insulin absorption (Figs. 1 and 4), suggesting the important roles of hyaluronidase-removable enzymes in insulin's metabolic loss during absorption. It has been shown that insulin was degraded by pancreatic proteases such as trypsin and chymotrypsin, which are believed to reside in both intestinal fluids and the mucous/glycocalyx layers (13,14). In this case, enzymes in the latter component could be removed as a result of the diminished mucous/glycocalyx layers by the hyaluronidase treatment. Meanwhile, the importance of ectopeptidases (e.g., aminopeptidases and dipeptidylpeptidases) contributing to the metabolic loss of certain polypeptides including insulin has been suggested recently (27–30). It is known that these enzymes are generally anchored with the epithelium and otherwise float as soluble forms (13,14,18,27–30), 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 4). In contrast, we have shown previously (3) 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·h), comparable to the 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 in insulin's metabolic loss following administration. Thus, as first proposed by Ugolev and Laey for relatively small polypeptides (31), it is likely that the intestinal membranes equip multiple 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 this hyaluronidase "perfusion" pretreatment are unique in the 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 (1–6) and are not specific enough to identify such (sub)cellular barrier compartments kinetically responsible for creating impediments and their physiologic regulation. In the latter aspect, it is quite interesting that the effects of hyaluronidase on insulin's ileal absorption appeared to depend on the "exposure" periods, as shown in Fig. 5. This leads to another speculation that the mucous/glycocalyx layers were reformed within a relatively short periods even in the presence of hyaluronidase "exposure" as their physiologic regulation. In fact, the intestinal glycocalyx has been shown to be replaced constantly by newly synthesized glycocalyx components, based on acetate-^{3}H monitoring (9). Heldin and Pertoft demonstrated that human mesothelial cells regained their coats in a full size within 5 h in culture following hyaluronidase pretreatment (32). Therefore, if this rapid turnover were the case, it would be reasonable that the magnitudes of the increasing effects of hyaluronidase "exposure" on insulin absorption were dependent on both concentration and "exposure" periods (Fig. 5). Nevertheless, as aimed at in the experimental design, the results from this "exposure" pretreatment would provide the feasibility of *in vivo* application of hyaluronidase preadministration in order to increase insulin absorption following oral administration without causing detectable cellular damage (i.e., the absence of LDH leakage).

CONCLUSIONS

Hyaluronidase pretreatment via "perfusion" and "exposure" successfully diminished the mucous/glycocalyx layers of the ileal epithelium without causing detectable damage in intra- and intercellular integrity. As a result, it was found that polypeptidic insulin's absorption was increased dramatically, whereas dextran's diffusive absorption was unaffected. Thus, its mechanisms should not be associated with cellular damage and diffusion but could be with reduced proteolytic activity, although these should be fully substantiated. Clearly, however, it has been first demonstrated that the preepithelial mucous/glycocalyx layers played a significant role in ileal absorption kinetics for certain macromolecules such as insulin. The increased insulin absorption seen following hyaluronidase "exposure" without detectable tissue damage would provide positive support for the usefulness of hyaluronidase *in vivo*, increasing insulin's bioavailability following oral administration.

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REFERENCES

1. V. H. L. Lee. *Peptide and Protein Delivery*. Marcel Dekker, New York, 1991.
2. K. L. Audus and T. J. Raub. *Biological Barriers to Protein Delivery.* Plenum Press, New York, 1993.
3. M. Morishita, I. Morishita, K. Takayama, Y. Machida, and T. Nagai. Site-dependent effect of aprotinin, sodium caprate, Na_2EDTA and sodium glycocholate on intestinal absorption of insulin. *Biol. Pharm. Bull.* **16**:68–72 (1993).
4. E. Ziv and M. Bendayan. Intestinal absorption of peptides through the enterocytes. *Microsc. Res. Tech.* **49**:346–352 (2000).
5. A. Yamamoto, T. Taniguchi, K. Rikyuu, T. Tsuji, T. Fujita, M. Murakami, and S. Muranishi. Effects of various protease inhibi-

tors on the intestinal absorption and degradation of insulin in rats. *Pharm. Res.* **11**:1496–1500 (1994).
6. T. L. Watts and A. Fasano. 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).
7. A. 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).
8. J. R. Pappenheimer. Role of pre-epithelial "unstirred" layers in absorption of nutrients from the human jejunum. *J. Membr. Biol.* **179**:185–204 (2001).
9. S. Ito. Structure and function of the glycocalyx. *Fed. Proc.* **28**:12–25 (1969).
10. A. Varki. Sialic acids as ligands in recognition phenomena. *FASEB J.* **11**:248–255 (1997).
11. G. Wang, G. Williams, H. Xia, M. Hickey, J. Shao, B. L. Davidson, and P. B. McCray. Apical barriers to airway epithelial cell gene transfer with amphotropic retroviral vectors. *Gene Ther.* **14**:922–931 (2002).
12. D. Massey-Harroche. Epithelial cell polarity as reflected in enterocytes. *Microsc. Res. Tech.* **49**:353–362 (2000).
13. J. F. Woodley and E. E. Sterchi. Endopeptidase activity of the small intestine. In: W. A. Hemmings (ed.) *Antigen Absorption by the Gut.* MTP Press Ltd., London, 1978, pp.199–205.
14. A. M. Ugolev, L. F. Smirnova, N. N. Iezuitova, N. M. Timofeeva, N. M. Mityushova, V. V. Egorova, and E. M. Parshkov. 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).
15. A. Rambourg and C. P. Leblond. Electron microscope observations on the carbohydrate-rich cell coat present at the surface of cells in the rat. *J. Cell Biol.* **32**:27–53 (1967).
16. O. N. Kovbasnjuk and K. R. Spring. The apical membrane glycocalyx of MDCK cells. *J. Membr. Biol.* **176**:19–29 (2000).
17. J. D. Esko. Proteoglycans and glycosaminoglycans. In: A. Varki, R. Cummings, J. Esko, H. Freeze, G. Hart and J. Marth (eds.). *Essentials of Glycobiology,* Cold Spring Harbor Laboratory Press, New York 1999, pp. 145–170.
18. J. P. F. Bai. 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).
19. C. B. S. Henry and B. R. Duling. Permeation of the luminal capillary glycocalyx is determined by hyaluronan. *Am. J. Physiol.* **277**:H508–H514 (1999).
20. V. H. Huxley and D. A. Williams. Role of a glycocalyx on coronary arteriole permeability to proteins: evidence from enzyme treatments. *Am. J. Physiol.* **278**:H1177–H1185 (2000).
21. R. D. Ennis, L. Borden, and W. A. Lee. 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).
22. E. S. Swenson, W. B. Milisen, and W. Curatolo. Intestinal permeability enhancement: efficacy, acute local toxicity, and reversibility. *Pharm. Res.* **11**:1132–1142 (1994).
23. M. Bendayan, E. Ziv, D. Gingras, R. Ben-Sasson, H. Bar-On, and M. Kidron. 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).
24. J. J. M. Bergeron, R. Rachubinski, N. Searle, D. Borts, R. Sikstrom, and B. I. Posner. Polypeptide hormone receptors *in vivo. J. Histochem. Cytochem.* **28**:824–835 (1980).
25. M. E. Forgue-Lafitte, M. R. Marescot, M. C. Chamblier, and G. Rosselin. Evidence for the presence of insulin binding sites in isolated rat intestinal epithelial cells. *Diabetologia* **19**:373–378 (1980).
26. M. D. Fernandez-Moreno, M. Serrano-Rios, and J. C. Prieto. Identification of insulin receptors in epithelial cells from duodenum, jejunum, ileum, caecum, colon and rectum in the rat. *Diabete Metab.* **13**:135–139 (1987).
27. Y. S. Quan, T. Fujita, D. Tohara, M. Tsuji, M. Kohyama, and A. Yamamoto. Transport kinetics of leucine enkephalin across Caco-2 monolayers: quantitative analysis for contribution of enzymatic and transport barrier. *Life Sci.* **64**:1243–1252 (1999).
28. B. Forbes, C. G. Wilson, and M. Gumbleton. Temporal dependence of ectopeptidase expression in alveolar epithelial cell culture: implications for study of peptide absorption. *Int. J. Pharm.* **180**:225–234 (1999).
29. F. Y. Liu, D. O. Kildsig, and A. K. Mitra. Pulmonary biotransformation of insulin in rat and rabbit. *Life Sci.* **51**:1683–1689 (1992).
30. L. L. Chang and J. P. F. Bai. Evidence for the existence of insulin-degrading enzyme on the brush-border membranes of rat enterocytes. *Pharm. Res.* **13**:801–803 (1996).
31. A. M. Ugolev and P. D. Laey. Membrane digestion. A concept of enzymic hydrolysis on cell membranes. *Biochim. Biophys. Acta* **300**:105–128 (1973).
32. P. Heldin and H. Pertoft. Synthesis and assembly of the hyaluronan-containing coats around normal human mesothelial cells. *Exp. Cell Res.* **208**:422–429 (1993).