Evidence for the Existence of Insulin-Degrading Enzyme on the Brush-Border Membranes of Rat Enterocytes

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Received November 6, 1995; accepted February 16, 1996
KEY WORDS: brush-border membranes; insulin-degrading enzyme; oral insulin.

INTRODUCTION

The brush-border membrane is the outermost lining of intestinal epithelium, hosting a battery of endo- and exopeptidases to efficiently degrade peptides (1). Such a powerful digestive feature makes it an obstacle to oral delivery of peptide and protein drugs. Insulin, being a large peptide, exhibits very low oral bioavailability due to its extensive proteolysis in the gastrointestinal tract. It is not only digested by luminal enzymes, but hydrolyzed by mucosal homogenates (2). It has been reported that insulin-degrading enzyme (IDE) is present in intestinal enterocytes, with its activity localized to the cytosol (2–4). However, to date, degradation of insulin by brush-border enzymes is not clear.

Extensive studies on insulin metabolism by its target cells have shown that the hormone is rapidly degraded by IDE, a nonlysosomal thiol metalloprotease (5). This enzyme, though mainly residing in the cytosol of insulin-target cells, was also proposed to be present on the plasma membranes of hepatocytes, kidney cells, muscle cells, and lymphocytes (6–9). Evidence supporting this hypothesis is based on the similarity between insulin degradation by plasma membranes and by IDE.

Though the transport mechanism of insulin through intestinal epithelium is not clear, transcellular transport has been suggested (10, 11). Since brush-border membranes account for the largest surface portions of intestinal epithelium, insulin will be exposed to their enzymes regardless of its absorption pathway(s) before being transported into the circulation. Hence understanding degradation by brush-border enzymes can not be overemphasized. In this study, anti-IDE antibody was used to identify the existence of IDE on the brush-border membrane and to determine its contribution to insulin degradation in an attempt to verify the significance of IDE in limiting oral absorption of insulin.

MATERIALS AND METHODS

Materials

N-Ethylmaleimide, 1,10-phenanthroline, protein G-sepharose CL-4B and pentobarbital were purchased from Sigma (St. Louis, MO); ^{125}I-(A14)-human recombinant insulin, peroxidase-conjungated sheep anti-mouse IgG, rainbow molecular weight markers and ECL detection system from Amersham (Arlington Heights, IL); protein assay kits, and gel reagents from Bio-Rad (Richmond, CA). Mouse monoclonal antibody 9B12 to human erythrocyte IDE and antibody $C_{20-3.1A}$ to rat IDE were gifts of Dr. Richard A. Roth (Stanford University Medical Center, Stanford, CA) (12) and Dr. William C. Duckworth (University of Nebraska, Omaha, NE), respectively. All other reagents were of analytical grade. All chemicals were used as obtained.

Methods

Preparation of Brush-Border Membrane Vesicles (BBMVs)

Thirteen male Sprague-Dawley rats, 300–350 g, were sacrificed with an overdose of pentobarbital. The jejunum, ileum, and proximal colon were obtained and washed with ice-cold saline. Brush-border membrane vesicles were prepared by the calcium precipitation method as published previously (13). Briefly, the mucosal cells were scraped off, and homogenized in a buffer containing 50 mM mannitol, and 2mM Hepes/Tris (pH7.5). $CaCl_2$ was added to the homogenates to achieve a final concentration of 10 mM, and the solution was stirred for 10 min. The mixture was centrifuged at 3,000× g for 15 min, and then at 27,000× g for 30 min. Pellets were resuspended in Tris/HCl buffer (50 mM Tris, 125 mM NaCl, pH 7.5) and homogenized with a glass/Teflon Potter homogenizer. Purified brush-border membranes were obtained by repeating the centrifugations at 3,000× g and 27,000× g. Protein concentrations were determined using the Bradford method . Activities of enzymes, alkaline phosphatase (a brush-border membrane marker enzyme) and lactate dehydrogenase (a cytosol marker), were determined to ensure the quality of the preparations.

Insulin Degradation

Proteolysis of insulin was assayed using the TCA (trichloroacetic acid) method as previously described (4). In brief, 30 pM ^{125}I-(A14)-insulin was incubated with brush-border membrane vesicles in the presence or absence of various inhibitors at 37C. At predetermined times, the reaction was stopped with 10% (w/v) TCA and centrifuged for 2 min. The extent of degradation was determined from the TCA-soluble radioactivity.

Immunoblotting

Brush-border membrane proteins from jejunum, ileum and colon were solubilized in 0.1% Triton X-100 and electrophoresed on reducing 7.5% polyacrylamide/sodium dodecyl sulfate gel (SDS-PAGE) and then transferred to a nitrocellulose membrane. The membrane was blocked with 10% (w/v) nonfat milk in Tris-Tween buffer. After probing with anti-IDE antibody 9B12 (12) for 2 hr at room temperature, the membrane was further incubated with peroxidase-conjugated sheep anti-mouse IgG (1:1000) for 1 hr. The bands were detected using the Amersham's ECL detection system.

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Immunoprecipitation

Ileal brush-border membrane proteins were solubilized in 0.1 % Triton X-100 for 30 min at 4°C. The solubilized proteins were incubated with the monoclonal antibody to human erythrocyte IDE for 16 hours at 4°C. The immunocomplex was then precipitated by incubating with protein G-sepharose beads for 1 hour at 4°C. The resulting supernatant after centrifugation was subjected to insulin degradation assay.

RESULTS AND DISCUSSION

The purity of BBMVs was confirmed by the enrichment factor for alkaline phosphatase: 16.3 for jejunal BBMVs, 8.9 for ileal BBMVs, and 12.9 for colon BBMVs. Since the cytosol of enterocytes has significant insulin-degrading activity (IDA), possible contamination in the preparation from cytosol was examined. Results showed that the activity ratios of BBMVs to homogenate for lactate dehydrogenase (a cytosolic marker) were 0.03, 0.01, and negligible in jejunum, ileum, and colon, respectively. This trace activity indicates contamination from cytosol was minimal.

Degradation of insulin by the brush-border membranes at 30 pM is shown in Table 1. Insulin-degrading activity (IDA) is highest in ileal BBMVs, followed by jejunal BBMVs, and then colon BBMVs. The distribution of insulin-degrading activity along the rat intestinal tract seems to correlate with that of insulin receptors throughout the intestine, with more receptors in the small intestine than in the colon (14). Since the ratios of BBMVs to homogenates for IDE were significantly higher than those of LDH (0.17 vs. 0.03 and 0.22 vs. negligible in ileum and colon, respectively), the contribution of cytosolic IDE to brush-border IDA was considered minor. Evidence provided here indicates that brush-border enzymes are capable of degrading large molecules like insulin. This is also supported by what was observed by others (15).

IDA in the pH range of 4.5–8.5 was tested in the jejunal BBMVs. The activity was maximal at pH 7.5, and was negligible at pH 4.5. This pH dependent profile conforms to the general feature of brush-border proteolytic activity and precludes any contaminated lysosomal proteolytic activities. Table 2 summaries the inhibitory effects of two potent IDE inhibitors, N-ethylmaleimide (NEM) and 1,10-phenanthroline, on brush-border IDA. Both inhibitors showed significant but not complete inhibition, suggesting the requirements for free thiol group and metal ion. The results imply the possible presence of IDE on the brush-border membranes.

Western blot analysis provided further evidence for the presence of IDE on the brush-border membranes. As shown in Fig. 1, a major band with an apparent M_r of 110 kD reacting with the IDE monoclonal antibody on the reducing gel was seen in purified BBMVs from jejunum, ileum and colon. Since contamination by cytosol IDE has been ruled out as discussed above, the results indicate the existence of IDE on the BBM of rat enterocytes.

In an immunoprecipitation study of solubilized ileal brush-border proteins, the antibody 9B12 removed 47.6 ± 4.9 % of insulin-degrading activity, but the normal mouse IgG did not show any significant effects (Fig. 2). The removal of insulin-degrading activity by IDE antibody suggests that IDE participates in the *in vitro* proteolysis of insulin. The participation of IDE in brush-border degradation of insulin was further supported by the observation that another monoclonal anti-IDE antibody, $C_{20\text{-}3.1A}$, which has weak metabolic inhibition on insu-

Table I. Distribution of Brush-border Insulin-degrading Activity (IDA) Along the Rat Intestine

Segment	IDA (pmole/min/g protein)
Jejunum	1.08 ± 0.03
Ileum	1.36 ± 0.13
Colon	0.78 ± 0.05

Note: Results are means ± SE of three experiments.

Table II. Effects of Inhibitors on Insulin Degradation by Jejunal, Ileal, and Colonic Brush-border Membranes

Inhibitors	% Inhibition		
	Jejunum	Ileum	Colon
N-Ethylmaleimide (2 mM)[a]	77.3 ± 5.0	77.4 ± 3.8	97.7 ± 1.4
1,10-Phenanthroline (1 mM)[b]	45.1 ± 8.2	74.7 ± 2.8	32.7 ± 8.6

[a] from six experiments.
[b] from three experiments.
Note: Results are expressed as mean ± SE.

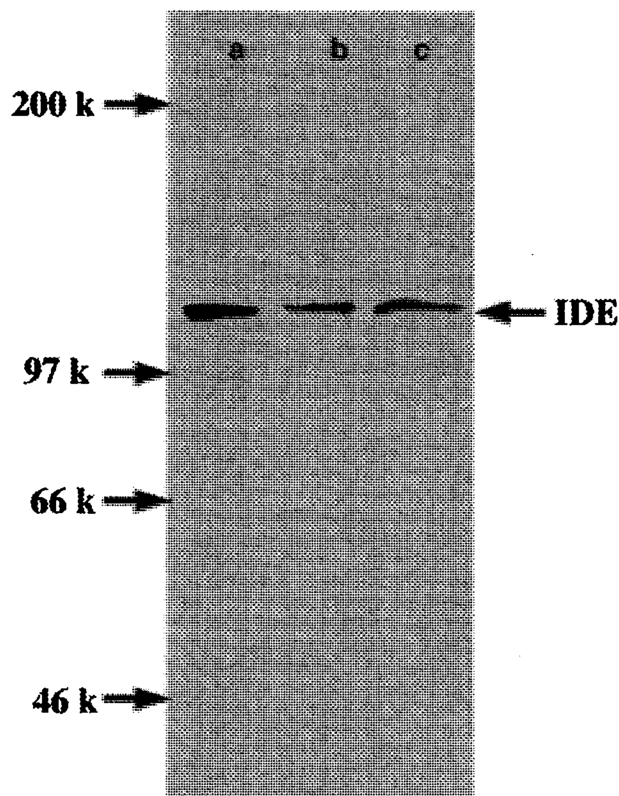

Fig. 1. Western blot analysis of intestinal brush-border membranes. Lane a: ileal BBM; lane b: colonic BBM; lane c: jejunal BBM.

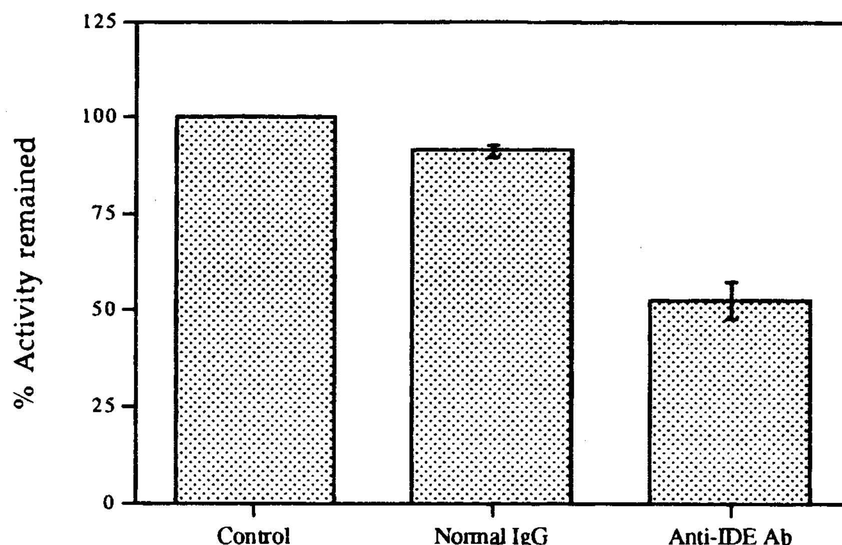

Fig. 2. Insulin-degrading activity of solubilized ileal brush-border proteins after immunoprecipitation by monoclonal anti-IDE antibody 9B12. The ratio of membrane protein to antibody is 20:1. Error bars are the standard errors of three experiments.

lin degradation by rat hepatocyte IDE (communication with Dr. Duckworth), diminished ileal brush-border and cytosolic IDA to a similar extent (17.3 ± 3.6 and 18.3 ± 0.5% of inhibition, respectively).

In conclusion, rat intestinal brush-border membranes are capable of degrading insulin, and IDE exists on the brush-border membranes and participates in *in vitro* insulin degradation. Since the brush-border membranes will confront insulin molecules, regardless of its absorption mechanism, these findings point to the significance of IDE in limiting the oral absorption of insulin.

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