Transepithelial Transport of Insulin: I. Insulin Degradation by Insulin-Degrading Enzyme in Small Intestinal Epithelium

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Received October 18, 1994; accepted March 25, 1995

Purpose. The purpose of this study was to determine the existence of insulin-degrading enzyme (EC 3.4.22.11) (IDE) in rat intestinal enterocytes. **Methods.** Subcellular fractionation, biochemical characterization, immunoprecipitation, and western blots were employed. **Results.** Insulin-degrading activity was localized in the cytosol, constituting 92% of total insulin-degrading activity. Cytosolic insulin-degrading activity had a pH optimum of 7.5, was almost completely inhibited by IDE inhibitors (N-ethylmaleimide, 1,10-phenanthroline, EDTA, p-chloromercuribenzoate, bacitracin), but was not or only weakly inhibited by others (aprotinin, chymostatin, leupeptin, and diisopropyl phosphofluoridate.) Further, cytosolic insulin-degrading activity had a Km of 78 nM, sharing a similar Km value with insulin-degrading enzyme in non-purified forms. Approximately, 87 ± 1.7% of cytosolic insulin-degrading activity was removed by the monoclonal antibody to IDE. On the SDS gel, the molecular weight of cytosolic IDE was 110 KD which is the same as that of human IDE. **Conclusions.** IDE is the major enzyme which degrades insulin in enterocytes.

KEY WORDS: insulin-degrading enzyme; monoclonal antibody to IDE; IDE inhibitors.

INTRODUCTION

The oral absorption of insulin into the portal vein mimics the endogenous secretion of insulin. Moreover, in animals, small chronic doses of oral insulin have been demonstrated to prevent the onset of autoimmune insulin-dependent diabetes mellitus (1). Therefore, the delivery of insulin via the oral route has been an ongoing interest.

There are three major barriers limiting oral insulin absorption: lumenal degradation, aggregation and degradation in absorptive cells. Lumenal degradation and insulin aggregation have been characterized (2–4), but not much is known about insulin degradation in intestinal enterocytes. Insulin is degraded by intestinal mucosal homogenates (5), revealing that transepithelial absorption of insulin is limited by enzymatic degradation, but the enzymes involved are unknown.

Insulin-degrading enzyme (IDE) (EC 3.4.22.11) has been well characterized in insulin target tissues and cells (6). In these cells, IDE, localized in the cytosol, is the enzyme which has been suggested to initiate insulin metabolism after insulin binds to the receptor (6). IDE, a neutral thiol metalloproteinase with a Km of 22 to 40 nM for the purified enzyme and of an order of magnitude higher for the non-purified forms, is present in the liver, adipocytes, muscle cells, erythrocytes, kidney, and other cells (6,7). It is also present in the intestine (8), but whether this enzyme is present in intestinal mucosal cells is unknown. Importantly, insulin receptor is present on the membrane of intestinal enterocytes of various species and Caco-2 cells (9–11), and insulin is internalized into rat intestinal enterocytes (12). The rat is often used as an animal model for the evaluation of drug absorption and disposition. This study was conducted in rats to test the hypothesis that IDE is the major enzyme responsible for the degradation of insulin in intestinal mucosal cells.

MATERIALS AND METHODS

Materials

Bovine serum albumin, aprotinin, trichloroacetic acid, protein G and pentobarbital, bacitracin, 1,10-phenanthroline, N-ethylmaleimide, EDTA, p-chloromercuribenzoate, chymostatin, soybean trypsin inhibitor, leupeptin, aprotinin, and Dip-F (diisopropyl phosphofluoridate) were obtained from Sigma Chemical Co. (St Louis, MO). The mouse monoclonal antibody to human RBC IDE was a gift from Dr Richard A. Roth (Department of Pharmacology, University of California, CA). Ovalbumin, bovine albumin, phosphorylase b, and myosin, and ^{125}I-(A14)-human recombinant insulin were obtained from Amersham Corporation (Arlington Heights, IL). Zinc-free human recombinant insulin was a gift from Dr. John Wang (Scios Nova Inc., CA). Dye regent and bovine γ-globulin for the protein assay, SDS gel, and nitrocellulose membranes were obtained from Bio-Rad Lab. (Richmond, CA). All other chemical reagents and buffer components were of analytic grade. All chemicals were used as obtained.

Methods

Male Sprague-Dawley rats, 250–300 g, were used as the animal model. The number of measurement was 3 for each experiment and the reported data represent mean ± S.E.

Degradation by Subcellular Fractions

The abdomen was opened by a mid-line incision after the rat was killed by an overdose of pentobarbital. The small intestine was cut longitudinally to expose the mucosal surface, and mucosal tissues were scraped off. One gram mucosal tissue was suspended in 10 ml 50 mM Tris/HCl buffer (pH 7.5) and 125 mM NaCl, and homogenized by a motor-driven glass/Teflon Potter homogenizer in an ice bath with 10 strokes at the speed of 1140 rev min. Mucosal homogenate was subject to a series of differential centrifugations at 4°C: 10000 g for 20 min, 27000 g for 30 min, and 100,000 g for 1 hr (13,14). The protein concentration of each subcellular fraction was determined using the Bradford method (15). Enzyme markers for Golgi membrane (α-d-mannosidase), mitochondria (succinate dehydrogenase), and cytosol (lactate dehydrogenase) were assayed (16–18), as published elsewhere, to confirm the preparation of subcellular fractions (16).

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0724-8741/95/0800-1171$07.50/0

Insulin-degrading activities in homogenate and cytosol were determined using the TCA (trichloroacetic acid) method (19,20). The incubation mixture consisted of 50 mM Tris/HCl buffer (pH 7.5 at 37°C), 1% BSA (w/v), 125 mM NaCl, 30 pM ^{125}I-(A14)-insulin, and 100 to 600 μg/ml of homogenate or cytosol protein (20). Incubation periods were 0, 1, 5, and 10 min. The 200 μl 15% TCA was added to 200 μl of incubation mixture to stop proteolysis. The final TCA concentration and pH in the mixture were 7.5% and less than 1, respectively. The resulting mixture was then centrifuged at 3,000 X g for 10 min. TCA was used to precipitate intact insulin while the degraded insulin fragments remained soluble in TCA solution. Radioactivity of the supernatant containing soluble insulin fragments was counted using a γ-counter. The degraded insulin was then estimated from the standard curve of insulin standard solutions; specific activity (pmol/mg protein/min) was obtained from linear regression. It was found that the hydrolysis rates obtained were similar using a final concentration of 7.5% or 2.5% of TCA. In the control, TCA was added to the insulin solution before subcellular protein was added.

pH Optimum, Effects of Inhibitors, and Degradation Kinetics

The pH optimum of cytosolic insulin-degrading activity was determined at 37°C, using 50 mM acetate buffer for pH 4.5 and 5.5, 50 mM phosphate buffer for pH 5.5, 6.5, 7.5, and 50 mM Tris buffer for pH 7.5 and 8.5. Each buffer also contained 125 mM NaCl. The effects of individual inhibitors on cytosolic insulin degradation were tested at 37°C. Individual inhibitors were 1,10-phenanthroline (1 mM), p-chloromercuribenzoate (0.2 mM), EDTA (5 mM), bacitracin (100 U/ml), N-ethylmaleimide (2 mM), soybean trypsin inhibitor (0.1 mg/ml), diisopropyl phosphofluoridate (1 mM or 0.1 mg/ml), leupeptin (0.1 mM), chymostatin (0.07 mg/ml), and aprotinin (0.5 mg/ml) (21–24). The first five inhibitors strongly inhibit IDE activity, and N-ethylmaleimide and p-chloromercuribenzoic acid also inhibit cytosolic proteasome (21,22), which has multicatalytic chymotrypsin-like, trypsin-like and cucumsin-like activities. Leupeptin, chymostatin, and aprotinin are strong inhibitors of proteasome (23,24). Leupeptin also inhibits lysosomal enzymes (25).

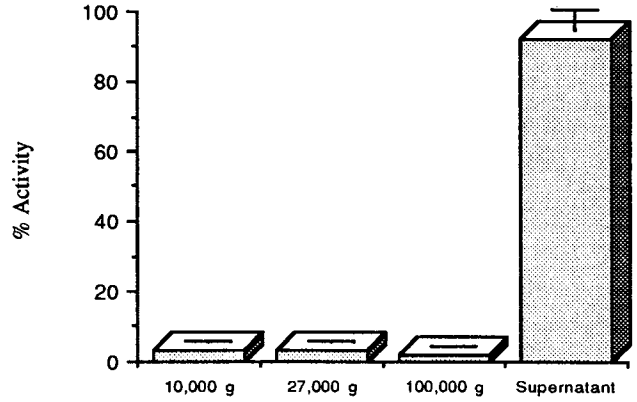

Fig. 1. The subcellular distribution of insulin-degrading activity in 10,000 X g pellet, 27,000 X g pellet, 100,000 X g pellet, and 100,000 X g supernatant (cytosol). N = 3 (Mean ± S.E.).

Table I. Effects of Inhibitors on Insulin-Degrading Activity in Cytosolic Fraction of Rat Small Intestine[a]

Inhibitors	Activity remained (%)
None	100.0
EDTA (5 mM)	45.2 ± 10
1,10-Phenanthroline (1 mM)	0
N-Ethylmaleimide (2 mM)	0
p-Chloromercuribenzoic acid (0.2 mM)	0
Bacitracin (100 U/ml)	0
Aprotinin (0.5 mg/ml)	91.6 ± 0.7
Trypsin inhibitor (0.1 mg/ml)	104.1 ± 4.7
Chymostatin (0.07 mg/ml)	76.3 ± 2.0
Diisopropyl-phospho-fluoridate (1 mM)	105.1 ± 8.3
Leupeptin (0.1 mg/ml)	103.9 ± 2.6

[a] Data are expressed as mean ± SE (n = 3).

Insulin degradation was studied at 30 pM, 10 nM, 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, and 1000 nM (20,26), using 30 pM labeled insulin plus varying amounts of nonlabeled insulin. The underlying assumption is that IDE does not distinguish labeled from nonlabeled insulin. The underlying assumption is that IDE does not distinguish labeled from nonlabeled insulin and recognizes both of them as the same substrate. The Henri-Michaelis-Menten equation is rearranged in Eq. 1, and the Michaelis constant, Km, is obtained from the double reciprocal plot.

$$1/V = \frac{Km}{Vmax}\frac{1}{[S]} + \frac{1}{Vmax} \quad (1)$$

Where Km is the Michaelis constant, Vmax is the maximal hydrolysis rate, and [S] is the total insulin concentration, i.e. labeled insulin plus nonlabeled insulin.

Immunoblotting and Immunoprecipitation

Immunoblotting was conducted using the method of Shii et al. 1986 (27). Briefly, cytosolic proteins were electrophoresed on the 7.5% polyacrylamide/NaDodSO$_4$ gel and transferred to the nitrocellulose membranes. The molecular

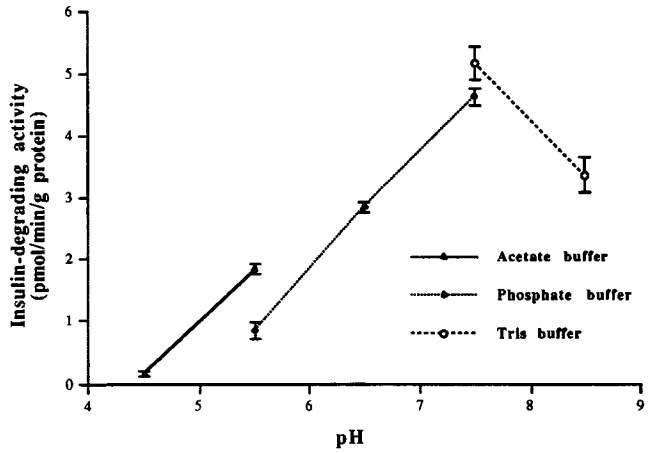

Fig. 2. The insulin-degrading activity/pH profile. N = 3 (Mean ± S.E.).

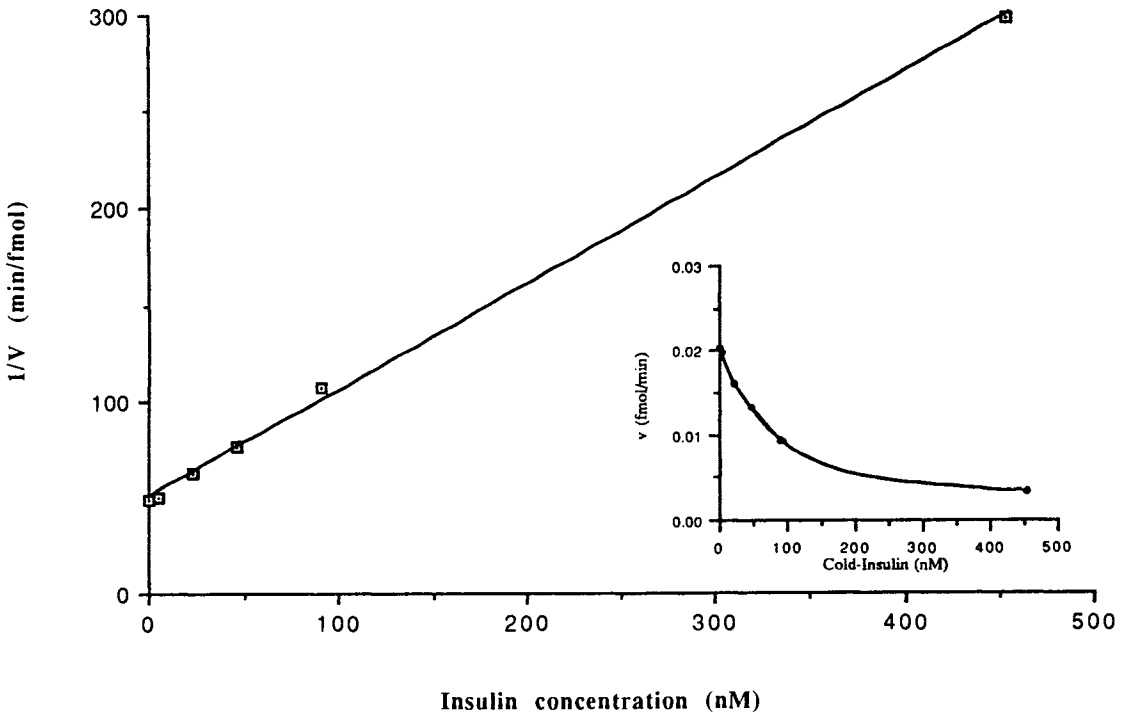

Fig. 3. The double reciprocal plot of degradation of 30 pM labeled insulin plus varying amount of nonlabeled insulin by mucosal cytosol.

weight markers were ovalbumin (46 K), bovine albumin (69 K), phosphorylase b (97 K), and myosin (200 K). The membranes were immunoblotted with anti-IDE monoclonal antibody (10 μg/ml) for 2 hr at 22°C, washed, and finally incubated with goat anti-mouse IgG antibody linked to horseradish peroxidase. After 2 hr at 24°C, the filters were washed, then bound immunoglobulin was visualized with a Bio-Rad horseradish peroxidase color development reagent.

Immunoprecipitation was conducted as published previously (27). Cytosolic protein (100 μg protein/ml) was incubated with the monoclonal antibody to IDE for 16 h at 4°C in a phosphate-buffered saline containing 0.2% BSA. The amount ratio of cytosolic protein and the monoclonal antibody to IDE was 10:1. In the control, normal mouse IgG was incubated with cytosolic protein in the same amount ratio. Then, protein G-sepharose CL-4B of 2 mg/ml was added to the incubation mixture to precipitate monoclonal antibody or normal mouse IgG, and then the mixture was centrifuged at 10,000 X g for 5 min. The resulting supernatant was tested for insulin-degrading activity.

RESULTS AND DISCUSSION

Specific insulin-degrading activities (pmole/min/g protein) were 0.49 ± 0.04 for 10,000 X g pellet, 0.65 ± 0.03 for 27,000 X g pellet, 0.73 ± 0.05 for 100,000 X g pellet, and 2.1 ± 0.14 for 100,000 X g supernatant. Considering the total amount of protein in each subcellular fraction, the cytosol had the highest percentage of insulin-degrading activity (Fig. 1). The subcellular distribution of insulin-degrading activity was similar to that of lactate dehydrogenase, a cytosol enzyme marker (16).

Though lysosomal enzymes might have leaked out during homogenization, the distribution of acid phosphatase and N-acetylglucosaminidase (75% in 10,000 X g pellet, 0% in 27,000 X g pellet and 100,000 X g pellet, and 23% in 100,000 X g supernatant) indicated minor contamination of lysosomal enzymes in the cytosol. Cytosolic insulin degradation was strongly inhibited by IDE inhibitors (N-ethylmaleimide, 1,10-phenanthroline, bacitracin, p-chloromercuribenzoic acid, and ETDA), but was not or very weakly inhibited by proteasome inhibitors and by lysosomal enzyme inhibitors (aprotinin, soybean trypsin inhibitor, chymostatin, leupeptin, and diisopropyl phosphofluoridate) (Table 1). Therefore, the role of lysosomal enzymes in cytosolic insulin degradation was likely minimal. The effects of inhibitors suggested that IDE-like activity was significantly involved in cytosolic insulin degradation.

In a preliminary study, three phosphate buffers of pH 6.5, 7.5 and 8 were studied, and it was found that insulin degrading activities were pH 7.5 > pH 8 > pH 6.5 (data not shown). Since the phosphate buffer has its buffer capability between pH 5 to pH 8, pH 8 is only 0.5 units higher than pH 7.5, and Tris buffer has a better buffer capability at pH 8.5 than phosphate buffer, Tris buffer was used instead, along with acetate and phosphate buffers, to establish a wider pH/activity profile. To ensure that the conclusion of pH optimum is not biased by the change of buffer species, insulin-degrading activities were determined in two buffers at the pH where one buffer was being changed to the other. The results of the preliminary screening test and of Fig. 2 confirmed that cytosolic insulin-degrading activity had a pH optimum of 7.5, similar to that of IDE.

Cytosolic insulin-degrading activity had a Km of 78 nM (Fig. 3), similar to that of IDE in non-purified forms (6,7). Since insulin aggregates at concentrations above 100 nM

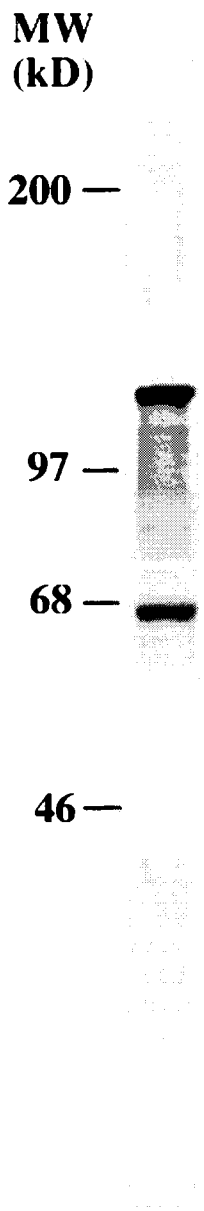

Fig. 4. Western blots using rat intestinal mucosal cytosol and monoclonal antibody to IDE. The molecular weight markers are ovalbumin (46 K), bovine albumin (69 K), phosphorylase b (97 K), and myosin (200 K).

(28), insulin should aggregate at 450 nM which was the highest concentration studied. According to Pekar and Frank, the association constants of zinc-free insulin, K_{dimer} and K_{hexmer}, at 25°C were 1.4×10^5 M^{-1} and 4×10^8 M^{-2}, respectively, in a pH 7.0 medium containing 0.1 M NaCl, 0.1 M Tris and 0.001 M EDTA (29). Importantly, the insulin monomer concentration increases as the total insulin concentration increases since aggregation/dissociation is an equilibrium process. In their condition, the majority of 450 nM insulin would be monomers with only 9% being dimers, i.e., the concentration of insulin monomers would be 410 nM. As compared to Pekar's and Frank's study, ionic strength was slightly lower, temperature was 12°C higher, and protein concentration was higher in our experimental condition. Hence, the degree of insulin aggregation in our study was unknown and could not be predicted based on the above-quoted association constants. It is, however, expected that the largest aggregate was dimers. Though forming dimers might affect insulin degradation, the linearity of the double reciprocal plot seemed to suggest that dissociation of insulin dimers to monomers was not a rate limiting step or that the concentration of insulin monomers in the system was well above the saturation level since for insulin degradation the Km was only 78 nM.

As shown in Fig. 4, a 110 KD protein reacted with the monoclonal antibody to IDE on the SDS gel, agreeing with the reported molecular weight of IDE on the reduced gel (7). Another protein of 68 KD also reacting with the monoclonal antibody to IDE was likely a degradation product of IDE. Approximately, 87 ± 1.7% of cytosolic insulin-degrading activity was removed by the monoclonal antibody to IDE when the amount ratio of cytosolic protein and monoclonal antibody ratio was 10:1. The normal mouse IgG did not remove any insulin-degrading activities, demonstrating that the binding between the monoclonal antibody to IDE and IDE is very specific. Had higher concentrations of monoclonal antibody to IDE been used, a higher extent of inhibition would have been observed. These two studies confirmed that IDE was present in small intestinal enterocytes and that it contributed to the majority of cytosolic insulin degradation.

This study, using biochemical characterization and immunological studies, identifies IDE as a very important enzyme that degrades insulin in small intestinal enterocytes. The results suggest that IDE activity may have to be inhibited in order to achieve useful insulin absorption in the intestine.

ACKNOWLEDGMENTS

This work was partially supported by AAPS New Investigator Grant. The authors would like to acknowledge T. Trinh, Phuong Tran and John Vu for their technical assistance.

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