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The Presence of Insulin-degrading Enzyme in Human Ileal and Colonic Mucosal Cells

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Abstract

The aim of this research is to characterize the presence of insulin-degrading enzyme in human colon and ileal mucosal cells. Biochemical studies, including the activity-pH profiles, the effects of enzyme inhibitors, immunoprecipitation and western blots, were conducted.

The majority of insulin-degrading activity in colon mucosal cells was localized in the cytosol. In both colon and ileum, cytosolic insulin-degrading activities had a pH optimum at pH 7.5, and were extensively inhibited by each of *N*-ethylmaleimide, *p*-chloromercuribenzoate, and 1,10-phenanthroline, but were very weakly affected by each of leupeptin, chymostatin, diisopropyl phosphofluoridate and soybean trypsin inhibitor. In the colon and ileum, more than 93% and 96%, respectively, of cytosolic insulin-degrading activities were removed by the mouse monoclonal antibody to human RBC insulin-degrading enzyme, as compared with less than 20% by the normal mouse IgG for both tissues. Further, a western blot analysis revealed that a cytosolic protein of 110 kD, in both human colon and ileum, reacted with the monoclonal antibody to insulin-degrading enzyme.

It is concluded that insulin-degrading enzyme is present in the cytosol of human colon and ileal mucosal cells.

Animal studies have suggested that oral chronic small doses of insulin can delay the onset of Type-I diabetes (Zhang et al 1991; Muir et al 1992). Currently, clinical trials throughout the USA, testing whether chronic oral administration of small doses of insulin can prevent diabetes are being planned, and are expected to begin in early 1996 (personal communication with NIH). This suggests that oral insulin is very important.

Biochemical and morpho-cytochemical evidence has suggested that insulin is mainly absorbed transcellularly through the ileum, duodenum and colon in normal and diabetic rats (Bendayan et al 1990, 1994). Using the adenocarcinoma cell line HT-29 derived from human colon, insulin was also shown to be absorbed by receptor-mediated endocytosis (Sonne 1985). It is, therefore, proposed that degradation by intestinal epithelium should be better understood in order to improve insulin absorption.

With fewer pancreatic enzymes, the ileum was noted to have higher permeabilities for several polypeptide drugs, including insulin (Lundin et al 1981; Lundin & Vilhardt 1986; Morishita et al 1993b); and the colon has negligible pancreatic enzymes (Bohe et al 1983). Most importantly, lumenal degradation of insulin by pancreatic enzymes and by microbial enzymes in the ileum and colon, respectively, can be minimized by non-absorbable Carbopol polymers (Bai et al 1995a). Further, insulin receptors are reportedly present on the cell membrane of colon and ileal enterocytes (Gallo-Payet & Hugon 1984; Pillion et al 1985). Therefore, the distal intestine, including the colon and ileum, is considered as the prime location for the oral delivery of insulin. Various systems for specific delivery to the distal intestine, taking advantage of the lumenal pH in the ileum and of the microbial enzymes in the colon, such as pectinolytic enzymes, amylase, dextrase, and azoreductase, have been reported (Saffran et al 1986; Bronsted & Hovgaard 1993; Milojevic et al 1993; Morishita et al 1993 a; Rubinstein et al 1993a). Though no studies have ever determined insulin proteolysis by human intestinal tissues, there have been several studies reporting degradation of insulin molecules by animal intestinal tissues (Atchison et al 1989; Yamamoto et al 1990).

Several in-vivo and in-vitro studies have suggested that insulin-degrading enzyme (IDE) (EC.3.4.22.11), an enzyme localized in the cytosol, is the major enzyme involved in intracellular metabolism of insulin in insulin target cells, such as hepatocytes (Hari et al 1987; Duckworth 1988). IDE is reportedly present in several rat tissues and Caco-2 cells, human colon adenocarcinoma cells (Hari et al 1987; Duckworth 1988; Kuo et al 1993; Bai & Chang 1995; Bai et al 1995), but has only been suggested to exist in human erythrocytes and lymphocytes (Roth et al 1985; Shii et al 1986). Utilizing the gene over-expression, the human gene encoding IDE was reported to be present in human chromosome 10 (Affholter et al 1990). However, it is not known whether IDE is expressed and present in the mucosal cells of the human intestine. The objective of this report is to examine the presence of IDE in human colon and ileal mucosal cells.

Materials and Methods

Chemicals

N-Ethylmaleimide, leupeptin, aprotinin, 1,10-phenanthroline, bacitracin, EDTA, *p*-chloromercuribenzoate, chymostatin, soybean trypsin inhibitor, trichloroacetic acid, Dip-F (diisopropyl phosphofluoridate), protein G and pentobarbital were obtained from Sigma Chemical Co. (St Louis, MO). The

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mouse monoclonal antibody to human RBC IDE was a gift from Dr Richard A. Roth (Department of Pharmacology, Stanford University School of Medicine, Stanford, CA). Protein assay kits, nitrocellulose membranes, bovine γ-globulin, and SDS gel were obtained from Bio-Rad Lab. (Richmond, CA). Bovine serum albumin (BSA), myosin, and ovalbumin, ^{125}I-(A14)-human recombinant insulin, and phosphorylase b was obtained from Amersham Corporation (Arlington Heights, IL). All chemical reagents and buffer components were of analytical (BSA) grade. All chemicals were used as obtained.

Insulin degradation in subcellular fractions

The use of human colon and ileal tissues was approved by the IRB: Human Subjects Committee of the University of Minnesota, according to federal guidelines 45 CFR Part 46.101. Fresh human intestinal tissues were rinsed and cleaned with ice-cold saline. Then mucosal tissues were scraped off and homogenized in saline using a glass/teflon potter homogenizer, and centrifuged at 108 000 *g* (Bai & Chang 1995). The protein concentrations and lactate dehydrogenase activities in homogenate and 108 000-*g* supernatant (cytosol) were determined as published previously (Bai & Chang 1995).

Insulin-degrading activities in homogenate and cytosol were determined using the TCA (trichloroacetic acid) method (Bai & Chang 1995). 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 mL^{-1} of homogenate or cytosolic protein (Bai & Chang 1995). Periodically 70 μL incubation mixture was taken and mixed with 250 μL TCA (10% w/v) to stop proteolysis. The final TCA concentration and pH in the mixture were 7.8% and less than 1, respectively. In general, a final TCA concentration below 10% was used (Duckworth 1990). The resulting mixture was then centrifuged at 6000 *g* for 10 min. Radioactivity of the supernatant containing soluble insulin fragments was counted using a γ-counter. The extent of degradation was then quantified using the standard curve of insulin standard solutions; specific activity (pmol (mg protein)$^{-1}$ min^{-1}) was obtained from linear regression. In the control, TCA was added to the cytosol or the homogenate before insulin solution was added.

pH optimum, effects of inhibitors and degradation kinetics

The pH-activity profiles of cytosolic insulin-degrading activity in ileal and colon cytosolic preparations were determined at 37°C, using 50 mM Tris buffer for pH 7·5 and 8·5, acetate buffer for pH 4·5 and 5·5, and phosphate buffer for pH 5·5, 6·5 and 7.5. NaCl (125 mM) was added to each buffer. Effects of enzyme inhibitors on cytosolic insulin degradation were tested at 37°C and pH 7·5 using a Tris buffer. Individual inhibitors and their concentrations in the incubation mixture were diisopropyl phosphofluoridate (1 mM) p-chloromercuribenzoate (0·2 mM), *N*-ethylmaleimide (2 mM), 1,10-phenanthroline (1 mM), leupeptin (0·1 mM), EDTA (5 mM), chymostatin (0·07 mg mL^{-1}), soybean trypsin inhibitor (0·1 mg mL^{-1}), and aprotinin (0·5 mg mL^{-1}) (Arrigo et al 1988; Atchison et al 1989; Duckworth et al 1989; Tsuji Kurachi 1989).

Insulin degradation kinetics were determined using 30 pM labelled insulin plus varying amounts of non-labelled insulin, as noted previously (Duckworth et al 1972; Yokono et al 1981). The underlying assumption is that IDE does not distinguish between labelled and non-labelled insulin and does recognize insulin in both forms, and that non-labelled insulin behaves as a competitive inhibitor. The data obtained were used for nonlinear regression using the rearranged Henri-Michaelis-Menten equation assuming K_m, the Michaelis constant, is the same as K_i, the inhibition constant, for nonlabelled insulin.

$$V = \frac{V_{max}\,[S]}{(K_m + [I]) + [S]} \quad (1)$$

Where V_{max} is the maximal hydrolysis rate, [I] is the non-labeled insulin concentration, K_m is the Michaelis constant, and [S] is the labeled insulin concentration.

Immunoblotting and immunoprecipitation

Western blots were performed as described previously (Bai & Chang 1995). Briefly, cytosolic proteins were electrophoresed on a 7·5% polyacrylamide/$NaDodSO_4$ gel and transferred onto the nitrocellulose membranes. The membranes were blocked, immunoblotted with anti-IDE monoclonal antibody (10 μg mL^{-1}) for 2 h at 22°C, washed, and then incubated with goat anti-mouse IgG antibodies which were linked to horseradish peroxidase. After 2 h at 22°C, bound immunoglobulin was visualized with a Bio-Rad horseradish peroxidase colour development reagent.

Immunoprecipitation was performed according to the method described by Shii & Roth (1986). Cytosolic protein was incubated with monoclonal antibody, for 16 h at 4°C, in phosphate-buffered saline containing 0·2% BSA. In the control, normal mouse IgG was used to replace the monoclonal antibody. The amount ratio of cytosolic protein and the monoclonal antibody (or normal mouse IgG) to IDE was 10:1. Then, protein G-sepharose CL-4B at 2 mg mL^{-1} was added to the incubation mixture to precipitate the monoclonal antibody, and then the mixture was centrifuged at 10 000 *g* for 5 min. The resulting supernatant was tested for insulin-degrading activity.

Results and Discussion

In the colon, insulin-degrading activity in the homogenate was 0·7 (0·02) pmol min^{-1} (g protein)$^{-1}$ while that in the cytosol was 1·05 (0·13) pmol min^{-1} (g protein)$^{-1}$, i.e., cytosolic insulin-degrading activity was 1·5 times that in homogenate. In this segment, 80% of insulin-degrading activity was localized in the cytosol, if taking into account the total insulin-degrading activity and protein concentration in each of homogenate and cytosol. The distribution of insulin-degrading activity in the cytosol was similar to that of lactate dehydrogenase, a cytosolic enzyme marker. The lactate dehydrogenase activity in the cytosol was 1·4 times that in the homogenate. Further, 84% of lactate dehydrogenase activity was localized in the cytosol. This result agrees with previous observations in that insulin-degrading activity was localized in the cytosol of rat intestinal mucosal cells and Caco-2 cells (Bai & Chang 1995; Bai et al 1995b). Because of the limited amount of human ileal tissue, the cytosol to homogenate insulin-degrading activity ratio was not determined; neither was the percentage of insulin-degrading activity present in the ileal cytosol. Since IDE was localized in the cytosol of rat intestinal mucosal cells (Bai & Chang

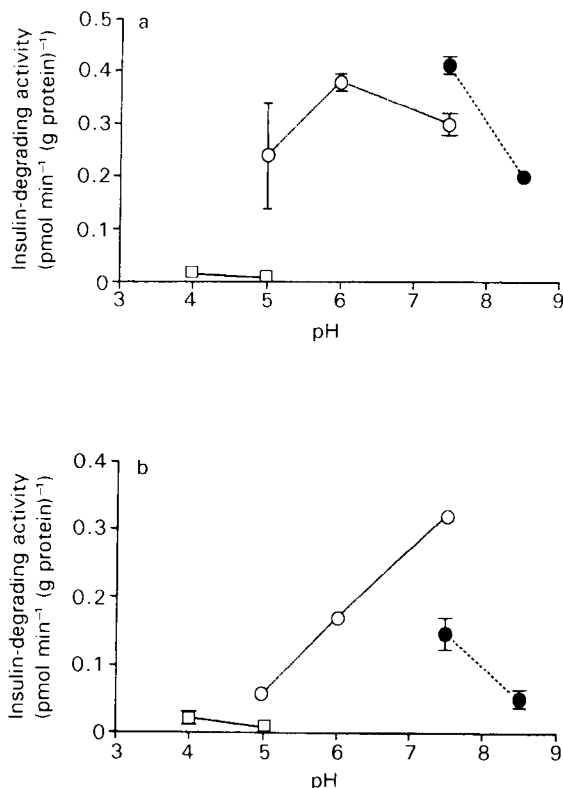

FIG. 1. Insulin-degrading activity-pH profiles. a. Ileal cytosol; b. colon cytosol in citrate (□), phosphate (○) or Tris (●) buffer.

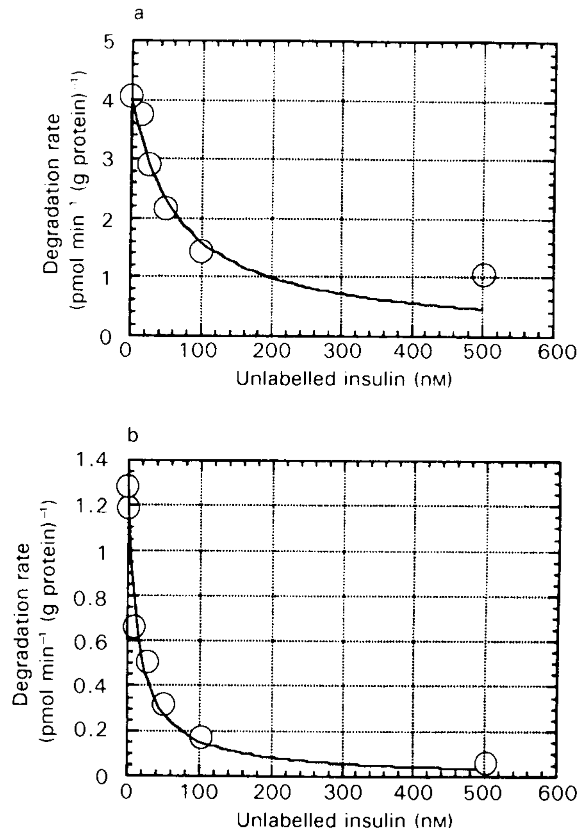

FIG. 2. Plots of insulin-degrading activity in the presence of various concentrations of unlabelled insulin. a. Ileal cytosol, b. colon cytosol.

1995) and insulin-degrading activity was mainly present in the cytosol of human colon mucosal cells, the presence of IDE in the cytosolic preparations of both human colon and ileum was determined.

The pH optimum, effects of inhibitors and degradation kinetics
As shown in Figs 1a and 1b, pH 7.5 was the pH optimum for cytosolic insulin-degrading activity in both colon and ileal mucosal cells, agreeing with the reported values for IDE (Shii et al 1986; Bai & Chang 1995). Since each buffer had its own optimum pH range, three buffers were used to determine the activity-pH profiles of cytosolic insulin-degrading activities. Interestingly, ileal insulin-degrading activity at pH 7.5 was higher in Tris buffer than in phosphate buffer while that in the colon was higher in phosphate buffer. Repeated experiments showed similar results. It is unknown why the colon insulin-degrading activity was higher in phosphate buffer than in Tris buffer. In previous studies using rat intestinal tissues, insulin-degrading activity in Tris buffer was always higher compared with that in phosphate buffer.

Table 1. Effects of enzyme inhibitors on cytosolic insulin-degrading activity in human intestinal mucosal tissues.

Inhibitor	Activity remaining (%)	
	Ileum	Colon
Control	100	100
N-Ethylmaleimide (2 nM)	3·1 ± 0·3	0·82 ± 2·3
1,10-Phenanthroline (0·2 nM)	51·9 ± 2·7	47·2 ± 4·1
p-Chloromercuribenzoate (2 nM)	0·3 ± 0·1	0
Bacitracin (100 units mL^{-1})	27·1 ± 0·8	N·D·
Diisopropylphosphofluoridate (1 nM)	91·6 ± 2·9	100 ± 10
Chymostatin (0·07 nM)	94·4 ± 4·4	87 ± 5·6
Soybean trypsin inhibitor (0·1 mg mL^{-1})	88·8 ± 6·6	81·4 ± 4·6
Leupeptin (0·1 mg mL^{-1})	82·9 ± 3·9	81 ± 3·7
Aprotinin (0·5 mg mL^{-1})	36·6 ± 3·4	38 ± 4·2

n = 3; data represent mean ± s.e. N.D. = not determined.

Table 2. Removal of cytosolic insulin-degrading activity in human intestinal mucosal tissues using immunoprecipitation.

Antibody	Activity remaining (%)	
	Ileum	Colon
Normal mouse IgG	84 ± 2·3	81 ± 1·5
Anti-IDE monoclonal antibody	3·2 ± 1·0	6·7 ± 0·9

n = 3; data represent mean ± s.e. The amount ratio of cytosolic protein to mouse IgG or to anti-IDE monoclonal antibody was 10:1.

The effects of enzyme inhibitors on cytosolic insulin-degrading activity are summarized in Table 1. Both ileum and colon cytosolic insulin-degrading activities were significantly inhibited by each of strong IDE inhibitors: *p*-chloromercuribenzoate, 1,10-phenanthroline, and *N*-ethylmaleimide (Shii et al 1986). Bacitracin also extensively inhibited ileal insulin-degrading activity. Other inhibitors, such as leupeptin, chymostatin, diisopropyl phosphofluoridate and soybean trypsin inhibitor, only very weakly inhibited colon and ileal cytosolic insulin-degrading activities. Since leupeptin and chymostatin are potent inhibitors of proteasome, an enzyme with multicatalytic activities (Arrigo et al 1988; Tsuji & Kurachi 1989), the results demonstrate that proteasome was unlikely to be significant in cytosolic insulin degradation. Aprotinin, an inhibitor capable of inhibiting both proteasome and insulin-degrading enzyme, inhibited 60% of cytosolic insulin-degrading activities in both colon and ileum. Aprotinin at 0.5 mg mL^{-1} was reported to inhibit 50% of pure insulin-degrading enzyme activity (Roth et al 1985).

As shown in Figs 2a and 2b, the Michaelis constants of colon and ileal cytosolic insulin-degrading activities were 14 nM and 63 nM, respectively, falling in the reported range for IDE (Shii et al 1986; Bai & Chang 1995). In the literature, the reported K_m for purified IDE from lymphocytes was 30 nM (Shii et al 1986; Duckworth 1988). Considering the activity distribution in the colon cytosol, the effects of enzyme inhibitors, the pH optimum, and the Michaelis constant, the cytosolic insulin-degrading activities in both colon and ileum have the activity characteristics of insulin-degrading enzyme.

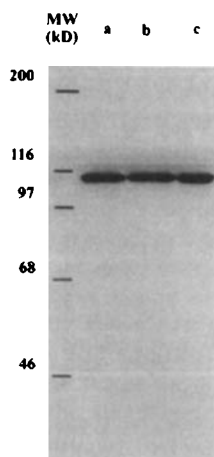

FIG. 3. Western blots analysis. a. Colon cytosol, b. ileal cytosol, c. Caco-2 cells cytosol. The molecular weight markers are ovalbumin (46 kDa), bovine albumin (69 kDa), phosphorylase b (97 kDa), and myosin (200 kDa).

Immunoprecipitation and Western blots

The presence of insulin-degrading enzyme in the cytosol of human colon and ileal mucosal cells was confirmed using a Western blots analysis. On the SDS-reduced gel, a protein of 110 kD was identified as IDE by the monoclonal antibody to IDE, in both colon and ileal cytosol preparations, as shown in Fig. 3. Similar results from a separate experiment comparing rat and human tissues were also observed (Chang et al 1996). The cytosol of Caco-2 cells, a human adenocarcinoma cell line in which IDE was identified in a previous report (Bai et al 1995b), was used as a reference. The molecular weight of IDE identified is consistent with the reported molecular weight (Shii & Roth 1986; Duckworth et al 1990; Bai et al 1995b). Interestingly, the molecular weight of IDE in rat intestine is similar to that in human intestine (Duckworth 1990).

As listed in Table 2, the monoclonal antibody directed against human RBC insulin-degrading enzyme was able to remove more than 90% of colon and ileal cytosolic insulin-degrading activities, while the normal mouse IgG removed less than 20% of cytosolic insulin-degrading activities. In previous studies using rat intestinal mucosal cells and Caco-2 cells, normal mouse IgG did not remove any cytosolic insulin-degrading activities (Bai & Chang 1995; Bai et al 1995b). Apparently, for human tissue there were some non-specific interactions between normal IgG and cytosolic insulin-degrading activities, accounting for the observed minor inhibition. Above all, after subtracting non-specific interaction, the result of immunoprecipitation still conclusively demonstrates that cytosolic insulin degradation is mainly due to IDE.

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