Insulin-Degrading Enzyme in a Human Colon Adenocarcinoma Cell Line (Caco-2)

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The activity of insulin-degrading enzyme (IDE), a thiol metalloprotease degrading insulin in many insulin target cells, was determined in human colon adenocarcinoma (Caco-2) cells. Insulin-degrading activity was localized in the cytosol of Caco-2 cells, accounting for 88% of total activity. Western blots and immunoprecipitation showed that IDE was present in the cytosol of Caco-2 cells and contributed to more than 93% cytosolic insulin-degrading activity. Cytosolic insulin degradation was strongly inhibited by IDE inhibitors, including *N*-ethylmaleimide, 1,10-phenanthroline, p-chloromericuribenzoate, and EDTA, but was not significantly or not as extensively inhibited by strong inhibitors of proteasome, i.e., chymostatin, soybean trypsin inhibitor, leupeptin, and Dip-F. These results suggest that IDE is present in Caco-2 cells, that Caco-2 IDE has properties similar to those of its counterparts in insulin-target tissues, and that it significantly contributes to intracellular insulin degradation.

KEY WORDS: Caco-2 cells; insulin-degrading enzyme.

INTRODUCTION

Small doses of oral insulin are being tested for efficacy in the prevention of autoimmune insulin-dependent diabetes mellitus (IDDM) (1). In animals, oral insulin has been shown to prevent or delay the onset of IDDM (2). To successfully prevent and treat IDDM, it is important to understand what barriers limit oral absorption of insulin in the intestine. Lumenal degradation, aggregation, and degradation in absorptive cells are the three major barriers for insulin absorption. Lumenal degradation and insulin aggregation have been characterized (3–5), but little is known about insulin degradation in intestinal enterocytes. Insulin was degraded by intestinal mucosal homogenates (6), indicating that enterocyte enzymes may limit transepithelial absorption of insulin. Effective inhibition of insulin degradation in the intestine and during absorption will be required to achieve effective oral absorption. Hence, it is essential to identify what enzyme is responsible for intracellular insulin degradation.

Insulin-degrading enzyme (IDE) (EC 3.4.22.11), localized in cytosol, is the cellular protease initiating insulin metabolism in many insulin target tissues and cells (7). IDE is a neutral thiol metalloproteinase, which is present in the liver, adipocytes, muscle cells, erythrocytes, kidney, and other cells (7,8). It has a K_m of 22 to 40 nM when purified, although the K_m is an order of magnitude higher in non-purified preparations (7,8). Recently, IDE has also been shown to exist in the kidney and intestine by identifying IDE mRNA (9), but it is unknown whether this enzyme is present in intestinal mucosal cells and whether it limits insulin absorption. After 14 days in culture, human colon adrenocarcinoma (Caco-2) cells develop mature microvilli and show characteristics of differentiated small intestinal mature epithelial cells (10). Further, the apical membrane of cultured Caco-2 cells has activities of brush-border membrane exo-/endopeptidases (11). Thus, Caco-2 cells provide a plausible model for in vitro evaluation and characterization of absorption and gut-wall metabolism of drugs (11,12). Importantly, insulin receptor was suggested to be present on the cellular membrane of Caco-2 cells, suggesting that insulin can be absorbed into the cells (13). This study was designed to understand whether IDE is present in Caco-2 cells and to determine if IDE accounts for the majority of insulin degradation in enterocytes.

MATERIALS AND METHODS

Materials

Bacitracin, 1,10-phenanthroline, *N*-ethylmaleimide, EDTA, *p*-chloromercuribenzoate, chymostatin, soybean trypsin inhibitor, leupeptin, aprotinin, Dip-F (diisopropylphosphofluoridate), and aprotinin, trichloroacetic acid, protein G and pentobarbital were obtained from Sigma Chemical Co. (St Louis, MO). Mouse monoclonal antibody to human RBC IDE was a gift from Dr Richard A. Roth (Department of Pharmacology, Standford University, California, CA). Ovalbumin, bovine albumin, phosphorylase b, myosin, and ^{125}I-(A14)-human recombinant insulin was obtained from Amersham Corporation (Arlington Heights, IL). Protein assay kits, bovine γ-globulin, 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

Insulin Degradation in Subcellular Fractions. Human colon adenocarcinoma (Caco-2) cells (American Type Culture Collection, Rockville, MD) were cultured, in an atmosphere of 4% CO_2, in tissue culture flasks using Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin. Caco-2 cells of less than 20 passages were used. After two weeks' culture the cells were detached from the flasks by adding 1 mM EDTA in phosphate-buffered saline and incubating for 10 min at 37°C. The cell suspension was centrifuged at 300 × g for 10 min, and the pellet was resuspended in pH 7.5 Tris buffer containing 125 mM NaCl. The cell suspension was homogenized mannually using a glass/teflon potter homogenizer and centrifuged at 108,000 × g (14). The protein concentrations of homogenate, 108,000 × g supernatant and cytosol were determined using the Bradford method (15).

Insulin-degrading activities in homogenate and cytosol

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were determined using the TCA (trichloroacetic acid) method (16,17). 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 cyotosolic protein (17). Periodically 200 μl of incubation mixture was taken and mixed with 200 μl 15% TCA (trichloroacetic acid) to stop proteolysis. The final TCA concentration and pH in the mixture were 7.5% and less than 1, respectively. According to the literature, if the final TCA concentration is above 5% large insulin fragments are TCA precipitable resulting in underestimation of hydrolysis (8). In general, a final TCA concentration below 10% is used (8). It was found that the hydrolysis rates in our experiments were similar using a final concentration of TCA at either 7.5% or 2.5%. The resulting mixture was then centrifuged at 6,000 × 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/min) was obtained from linear regression. In the control, TCA was added to the insulin solution before the subcellular protein was added.

pH optimum, Effects of Inhibitors and Degradation Kinetics. The pH optimum of cytosolic insulin-degrading activity in Caco-2 cells was determined at 37°C, using 50 mM acetate buffer for pH 4.5 and 5.5, phosphate buffer for pH 5.5, 6.5 and 7.5, and Tris buffer for pH 7.5 and 8.5. Each buffer also contained 125 mM NaCl. Effects of individual inhibitors on cytosolic insulin degradation were tested at 37°C and pH 7.5; Tris buffer was used for inhibition studies. Individual inhibitors and their concentrations in the incubation mixture were p-chloromercuribenzoate (0.2 mM), *N*-ethylmaleimide (2 mM), 1,10-phenanthroline (1 mM), EDTA (5 mM), soybean trypsin inhibitor (0.1 mg/ml), leupeptin (0.1 mM), chymostatin (0.07 mg/ml), aprotinin (0.5 mg/ml), and diisopropylphosphofluoridate (1 mM, or 0.1 mg/ml) (18–21).

Insulin degradation were studied over a concentration range, using 30 pM labeled insulin plus varying amount of nonlabeled insulin (16,22). The underlying assumption is that IDE does not distinguish between labeled and nonlabeled insulin and does recognize both of them as the same substrate. The Henri-Michaelis-Menten equation was rearranged, and the Michaelis-Menten parameters, V_{max} and K_m, were obtained from the Lineweaver-Burk plot assuming that the K_m of labeled insulin was equal to the K_i of nonlabeled insulin.

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

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

Immunoblotting and Immunoprecipitation. Immunoblotting was performed according to the method of Shii *et al.* (23). Briefly, cytosolic proteins were electrophoresed on 7.5% polyacrylamide/NaDodSO$_4$ gel and transferred to the nitrocellulose membranes. The membranes were blocked, immunoblotted first with anti-IDE monoclonal antibodies (10 μg/ml) for 2 hr at 22°C, washed, and then incubated with goat anti-mouse IgG antibodies linked to horseradish peroxidase. After 2 hr at 22°C, the filters were washed; bound immunoglobulin was visualized with Bio-Rad horseradish peroxidase color development reagent.

Immunoprecipitation was performed using the method of Shii et al. (23). Cytosolic protein (100 μg protein/ml) was incubated with monoclonal antibodies for 16 h at 4°C in phosphate-buffered saline containing 0.2% BSA. The amount ratio of cytosolic protein and the monoclonal antibody to IDE was 1:1. Then, protein G-sepharose CL-4B of 2 mg/ml was added to the incubation mixture to precipitate monoclonal antibodies, 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

A typical concentration/time profile of degraded insulin is shown in Figure 1, indicating that insulin degradation rate was appropriately obtained by linear regression. The subcellular distribution of insulin-degrading activity was estimated by considering activities per g protein in the cytosol and homogenate and the total amount of cytosolic and homogenate protein. Insulin-degrading activities in homogenate and cytosol were 16.5 and 23.6 pmole/min/mg protein, respectively. The total amount of cytosolic and homogenate protein were 10.6 and 6.5 mg, respectively, from 3.7 × 10^7 cells. Importantly, insulin-degrading activity was localized in the cytosol, constituting 88% of total insulin-degrading activity in homogenate. The results of insulin-degrading activity being localized in the cytosol of Caco-2 cells agrees with what was observed in many cell types using subcellular fractionation, including rat skeletal muscle, liver, kidney, brain and adipocytes and human erythrocytes (8,23–25).

It has been reported that IDE also is present in other organelles such as peroxisomes, which have higher concentration than the cytosol in transfected cell lines, Ltk$^-$ cells (26). It is unknown whether transfected cell lines have enhanced expression of IDE in peroxisomes compared to other cell types. Though, subcellular fractionation may result in the leakage of IDE from organelles into cytosol, some reports have localized IDE in the cytosol using immunolocalization methods (9,27). Further, the subcellular distribution of IDE in Caco-2 cells is similar to that of lactate dehy-

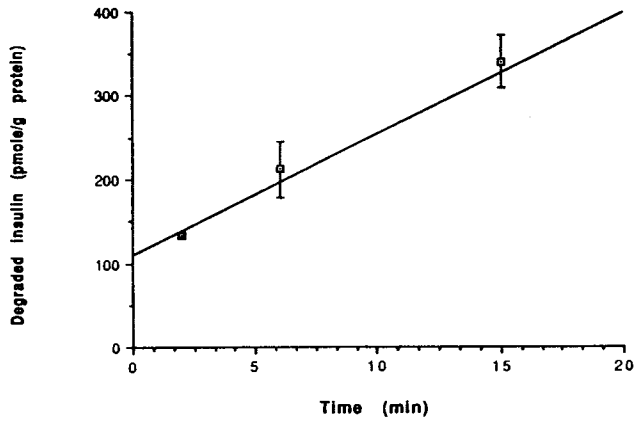

Fig. 1. Determination of rates of insulin degradation by the cytosol preparation from Caco-2 cells.

drogenase, a cytoplasm enzyme marker (data not shown). Though it is unknown how internalized insulin by endocytosis is released into cytosol, a cytosolic pathway of intracellular degradation of insulin has been confirmed (28). Hence, effects of inhibitors, pH optimum, kinetic studies, immunoblotting and immunoprecipitation were studied using the cytosol preparation.

Potentially, cytosolic IDE and proteasome may contribute to insulin degradation; the latter is a large enzyme with multicatalytic activities, i.e., chymostrypsin-like, trypsin-like, and cucumsin-like activities (20,21). Proteasome activity has been found in the cytosol of Caco-2 cells (data not shown.) Therefore, inhibitors of these two enzymes were tested. Each of *N*-ethylmaleimide, *p*-chloromercuribenzoate, 1,10-phenanthroline, and EDTA inhibited more than 80% of cytosolic insulin-degrading activity (Table I). Although these inhibitors are not specific IDE inhibitors, they have been shown to strongly inhibit IDE (8). The first two are sulfhydryl agents while the last two are chelators. Aprotinin, chymostatin, leupeptin, soybean trypsin inhibitor, and diisopropylphosphofluoridate (Dip-F) inhibited 57%, 11%, 0%, 14%, and 0% of cytosolic insulin-degrading activity, respectively (Table I). Aprotinin, chymostatin and leupeptin inhibit more than 90% of proteasome activity, but soybean trypsin inhibitor and Dip-F have weaker inhibition (20,21). Aprotinin at 0.5 mg/ml is also capable of inhibiting 50% of pure IDE activity (29). N-ethylmaleimide and p-chloromercuribenzoate are also inhibitors of proteasome, but EDTA and 1,10-phenanthroline have no inhibitory effect on proteasome (20,21). Leupeptin also inhibits lysosomal enzymes (30). Altogether, based on literature reports of inhibitor specificity, the results of effects of inhibitors suggest that IDE-like activity is responsible for insulin degradation in Caco-2 cells while proteasome-like activities plays only a minor role.

The presence of IDE in Caco-2 cytosol was confirmed by immunoblotting; a 110 KD protein reacted with the monoclonal antibody to human RBC IDE on the SDS gel (Fig. 2). This agrees with the molecular weight observed for IDE in various tissues using the same monoclonal antibody to IDE (23,31). The monoclonal antibody to IDE was developed using human RBC IDE, and has been shown to react with IDE in many tissues while normal mouse IgG has no reaction (23). Further, more than 92.8% of insulin-degrading activity in the cytosol was removed by the monoclonal antibody to IDE using immunoprecipitation (Fig. 3), conclusively demonstrating that IDE is responsible for the majority of insulin degradation in Caco-2 cytosol.

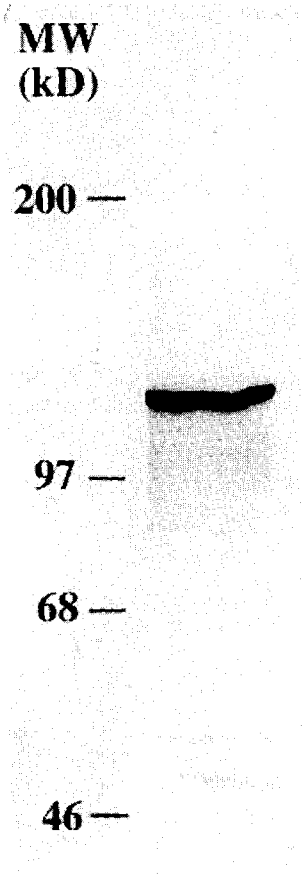

Fig. 2. Western blots using Caco-2 cytosolic protein and monoclonal antibody to IDE. Molecular weight markers, including ovalbumin (46K), bovine albumin (69K), phosphorylase (97K), and myosin (200K), are in the first column.

Table I. Effects of Enzyme Inhibitors on Insulin-Degrading Activity in the Cytosol Preparation of Caco-2 Cells

Inhibitors	% activity remained
None	100
N-Ethylmaleimide (2 mM)	6.4 ± 4.2
1,10-phenanthroline (0.2 mM)	18 ± 10
p-Chloromercuribenzoate (2 mM)	5.3 ± 3.5
EDTA (5 mM)	15 ± 3
Aprotinin (0.5 mg/ml)	44 ± 5
Leupeptin (0.1 mg/ml)	110 ± 6
Chymostatin (0.07 mg/ml)	92 ± 4
Diiisopropylphosphofluoridate (1 mM)	105 ± 5
Soybean trypsin inhibitor (0.1 mg/ml)	88 ± 2

The data represent the mean value of three experiments (mean ± SE).

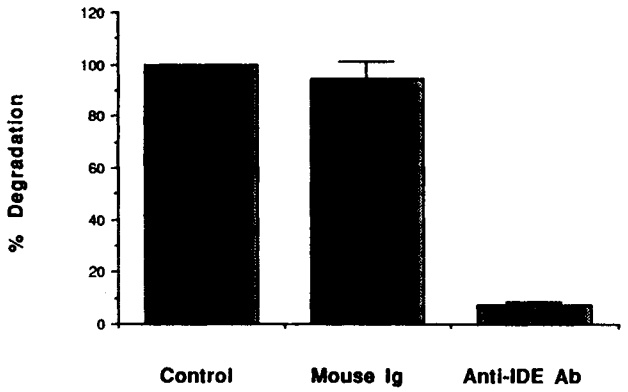

Fig. 3. Removal of insulin-degrading activity by immunoprecipitation using Tris, normal mouse IgG and anti-IDE monoclonal antibody. The weight ratio of cytosolic protein to IgG or to anti-IDE monoclonal antibody was 1:1.

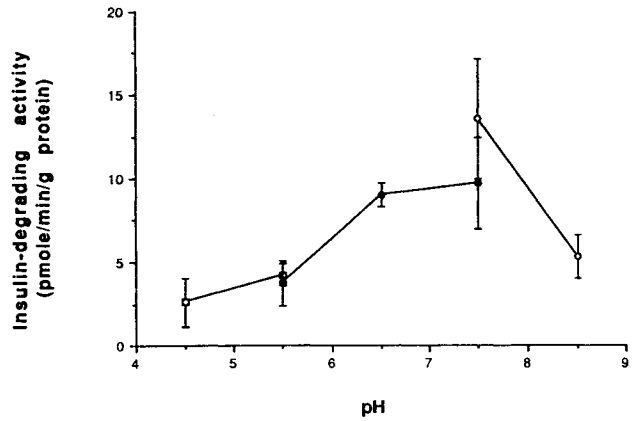

Fig. 4. The pH dependence of insulin-degrading activity in cytosol preparation.

Caco-2 cytosolic insulin-degrading activity had a pH optimum at 7.5 (Fig. 4), sharing the same pH optimum with IDE from the human and rat tissues (8). At pH 4.5 and pH 5.5, cytosolic insulin degradation was much slower. Though lysosomal enzymes may have leaked out during homogenization, they are active at acidic pH instead of alkaline pH. Further, leupeptin, also a lysosomal enzyme inhibitor which inhibits insulin degradation by lysosomes (30), did not have any effects on insulin degradation by cytosolic fractions at pH 7.5. This suggests that lysosomal enzymes do not play an important role in insulin in the cytosolic preparations. The Michaelis constant, K_m, was 100 nM (Fig. 5), indicating that Caco-2 IDE also shares a similar K_m value in the same range as IDE in other tissues and cells. The K_m of purified IDE is 20–40 nM, and that of nonpurified preparations is usually higher, ranging from 100–200 nM (22). The percentage of insulin-degrading activity remaining in the homogenate in the presence of N-ethylmaleimide, p-chloromercuribenzoate and 1,10-phenanthroline was 17.64 (2.0), 1.45(1.45), and 26.38 (3.58)%, respectively. Inhibition of homogenate insulin-degrading activity by *N*-ethylmaleimide and 1,10-phenanthroline was to a lesser extent than observed with cytosolic activity, but still substantial. The biochemical studies, including pH optimum, subcellular distribution, effects of enzyme inhibitors, degradation kinetics, and immunological studies (immunoblotting and immunoprecipitation), confirm that IDE is present in Caco-2 cells, localized in the cytosol, and is responsible for the majority of cytosolic insulin degradation. The implication of this study is that IDE activity has to be minimized in order to improve transepithelial transport of insulin.

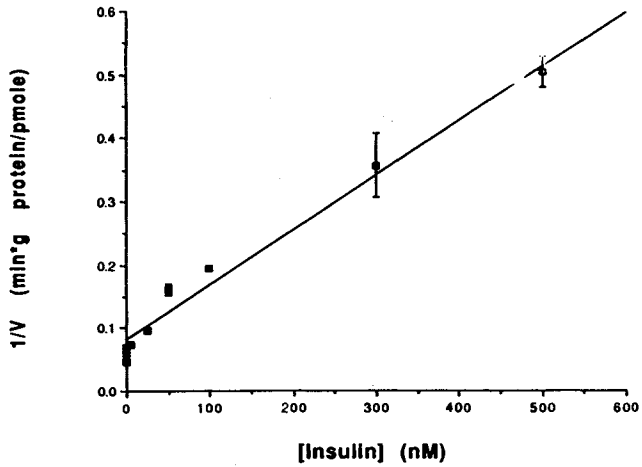

Fig. 5. Determination of the K_m for insulin of crude insulin-degrading enzyme in a cytosol preparation from the Caco-2 cells.

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