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The Involvement of Cytosolic Chymotrypsin-like, Trypsin-like, and Cucumsin-like Activities in Degradation of Insulin and Insulin-like Growth Factor I by Epithelial Tissues

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Abstract

Using specific substrates, benzyloxycarbonyl-Gly-Gly-Leu-*p*-nitroanilide, benzyloxycarbonyl-Gly-Gly-Arg-2-naphthylamide and benzyloxycarbonyl-Leu-Leu-Glu-2-naphthylamide, cytosolic chymotrypsin-like, trypsin-like and cucumsin-like activities were determined, respectively, in rat epithelial tissues and differentiated human Caco-2 cells.

The cytosolic fractions of rat colonic, rectal, nasal, and alveolar epithelial cells and differentiated human Caco-2 cells contained these three distinct enzyme activities. However, effects of enzyme inhibitors revealed that these three distinctive activities were not extensively involved in cytosolic or homogenate degradation of insulin and insulin-like growth factor I (IGF-I).

It is concluded that proteasome-like activities may not significantly limit nonparenteral absorption of peptide and protein drugs such as insulin and IGF-I.

Proteolytic degradation of peptide and protein drugs in various mucosal homogenates has been observed (Kashi & Lee 1986; Lee 1988), indicating that proteolytic degradation will potentially limit transepithelial transport of this class of compounds. On the other hand, cytosolic proteolytic activities can be utilized as the conversion enzymes for peptide prodrugs which are designed to improve oral absorption of drugs (Bai et al 1992).

A multicatalytic endopeptidase with three distinct activities, including chymotrypsin-like, trypsin-like, and cucumsin-like activities, has been reported in the cytosol of various tissues (Zolfaghari et al 1987; Yukawa et al 1991). This enzyme is ubiquitously present in eukaryotic cells. Proteasome has been suggested to be involved in the intracellular energy-dependent proteolysis of proteins. Different forms of proteasomes, 20 S and 26 S, seem to be responsible for distinct actions. The 20 S proteasome (700 kDa) is involved in degrading non-ubiquitinated proteins while the 26 S proteasome, a larger complex of proteasome, is involved in hydrolysing ubiquitinated proteins (Hoffman et al 1992). The proteasome probably plays an important role in regulating intracellular steady levels of proteins. The significance of proteasome in limiting nonparenteral absorption of peptide and protein drugs is unknown. The specific aim of this study is to determine the presence of chymotrypsin-like, trypsin-like, and cucumsin-like activities in colonic, nasal, rectal and alveolar epithelial cells and human Caco-2 cells, and to understand whether these three distinctive activities contribute to degradation of insulin and insulin-like growth factor I (IGF-I) by epithelial tissues. Since small intestinal mucosal tissues are subject to contamination by pancreatic enzymes, human Caco-2 cells, with morphological and biochemical characteristics similar to small intestinal epithelial cells (Hilgers et al 1990; Howell et al 1992), were used.

Materials

Benzyloxycarbonyl-Gly-Gly-Leu-*p*-nitroanilide, benzyloxycarbonyl-Gly-Gly-Arg-2-naphthylamide, benzyloxycarbonyl-Leu-Leu-Glu-2-naphthylamide, *p*-nitroaniline, foetal bovine serum, EDTA, trypsin, and EGTA were obtained from Sigma Chemical Co. (St Louis, MO). All buffer components were of analytical grade. Acetonitrile was of HPLC grade. All materials were used as obtained.

Preparations of pulmonary alveolar cells

Male Sprague-Dawley rats, 250–300 g, were used as the animal model. Alveolar epithelial cells were isolated as published previously (Kikkawa & Yoneda 1974). The rat was killed by an overdose of phenobarbitone; immediately, the trachea was cannulated with Teflon tubing. The lung was perfused with solution I (40 mL) via the right ventricle until it was free of blood. Solution I consisted of (mM): 140 NaCl, 5 KCl, 2·5 sodium phosphate, 10 HEPES, 2 $CaCl_2$, and 1·3 $MgSO_4$. The lung was removed from the chest and lavaged eight times with solution II (15 mL) via the trachea to remove macrophages at 37°C. Solution II consisted of (mM): 140 NaCl, 5 KCl, 2·5 sodium phosphate, 10 HEPES, 6 glucose, 0·2 EGTA, and 5 EDTA. Then 40 mL trypsin solution I (0·1 mg mL^{-1}) was instilled into the lung via the trachea under a constant pressure applied by the syringe for 30 min in a 37°C water bath. The trachea was detected; the whole lung was minced, using scissors, to 1-mm^3 pieces and incubated in trypsin solution for another 10 min. Foetal bovine serum (5 mL) was then added to the mixture to terminate the trypsin activity. The whole mixture was shaken in a water bath, at 37°C, at 130 cycles min^{-1} for 2 min, and centrifuged at 250 *g* for 20 min. The supernatant was discarded, and the pellet was resuspended in 50 mM Tris buffer (pH 7·5 at 37°C) containing 125 mM NaCl.

Centrifugation and resuspension were repeated three times to pellet alveolar epithelial cells.

Preparation of colonic, rectal and nasal cytosol

The colon and rectum were cut longitudinally to expose the mucosal surface (epithelium) and then mucosae from these tissues were scraped off. Nasal mucosa was prepared as published previously (Hong et al 1991). The skin was removed from the top of the skull and the nose, and the skull was split to expose the nasal cavity using a sharp knife. The nasal mucosa (including respiratory and olfactory epithelium) was stripped from the bone and cartilage of the nasal and paranasal cavities.

Preparation of Caco-2 cytosol

Human colon adenocarcinoma (Caco-2) cells (American Type Culture Collection, Rockville, MD) were cultured, under an atmosphere of 10% CO_2, in tissue culture flasks using Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum and penicillin-streptomycin (Hilgers et al 1990; Howell et al 1992). 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 (PBS) 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.

Enzyme activities

Human Caco-2 cells and colonic, rectal, nasal, and alveolar epithelial tissues were individually suspended in 10 mL 50 mM Tris/HCl buffer (pH 7·5) and 125 mM NaCl. Colonic, rectal, and nasal homogenates were homogenized by a glass/Teflon Potter homogenizer in an ice-bath, with 10 strokes, at the speed of 1140 rev min^{-1}. Human Caco-2 cells and alveolar epithelial cells were manually homogenized using a glass/Teflon Potter homogenizer. Homogenates were subjected to centrifugation at 100 000 *g* (1 h) (Bai 1993). The protein concentrations of homogenate and cytosol were determined as published previously (Bradford 1976).

Chymotrypsin-like, trypsin-like, and cucumsin-like activities were determined using specific enzyme substrates, benzyloxycarbonyl-Gly-Gly-Leu-*p*-nitroanilide, benzyloxycarbonyl-Gly-Gly-Arg-2-naphthylamide and benzyloxycarbonyl-Leu-Leu-Glu-2-naphthylamide, respectively (Zolfaghari et al 1987). Hydrolysis was performed as published previously (Bai 1993). The incubation mixture (300 μL) consisted of 50 mM pH 7·5 Tris/HCl buffer, NaCl (150 mM), a substrate, and homogenate or cytosol protein (0·1–2 mg). In the incubation mixture, the final concentrations of substrates were 1·25 mM for Z-Gly-Gly-Leu-*p*-nitroanilide, 1·4 mM for benzyloxycarbonyl-Leu-Leu-Glu-2-naphthylamide, and 1·6 mM for benzyloxycarbonyl-Gly-Gly-Arg-2-naphthylamide. Dioxane was used as the cosolvent to solubilize these substrates; its final concentration was 5%. Incubation was performed at 37°C. Samples were taken periodically and the reaction was stopped using 0·1 M HCl. In control, proteins in buffer were inactivated by HCl and then added to the substrate solutions, and no hydrolysis of any substrates was observed.

Degradation of insulin and IGF-I

Degradation of insulin and IGF-I by cytosol and homogenate was determined using the TCA (trichloroacetic acid) method (Duckworth et al 1972). 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 or 60 pM IGF-I, and 100–600 μg mL^{-1} of homogenate or cytosol proteins (Yokono et al 1981). Samples were taken at 0, 1, 5, 10, 30, and 60 min after incubation began. Two hundred microlitres 15% TCA was added to 200 μL incubation mixture to stop proteolysis; the resulting mixture was centrifuged at 13 000 *g* for 10 min. In control, TCA was added to the tissue before insulin or IGF was added. Radioactivity of the supernatant containing degraded insulin or IGF fragments was counted using a γ-counter. The degraded insulin and IGF-I were estimated from the standard curves of insulin and IGF-I, respectively. Specific activity was obtained from linear regression and expressed as pmol (mg protein)$^{-1}$ min^{-1}.

Effects of inhibitors

Effects of individual inhibitors on cytosolic insulin and IGF-I degradation were tested at 37°C. Individual inhibitors and their concentrations in the incubation mixture were leupeptin (0·1 mM), soybean trypsin inhibitor (0·1 mg mL^{-1}), diisopropylphosphofluoridate (1 mM), chymostatin (0·07 mg mL^{-1}), and aprotinin (0·5 mg mL^{-1}) (Arrigo et al 1988; Tsuji & Kurachi 1989). Leupeptin is also a strong inhibitor of lysosomal enzymes (Kirschke & Barrett 1987).

Assay

Analysis was by HPLC using an SIL autoinjector, an LC-600 pump, an SPD-6A UV spectrophotometric detector, a CR 601 recorder (Shimadzu Corporation, Kyoto, Japan), and a C_8 column (5 μm, 4·6 mm × 15 cm, Beckman Instrument, Berkley, CA). Mobile phases were acetonitrile: 0·005% TFA (31·5 : 68·5) for benzyloxycarbonyl-Leu-Leu-Glu-2-naphthylamide, and acetonitrile : 0·005% TFA (34·5 : 65·5) for benzyloxycarbonyl-Gly-Gly-Arg-2-naphthylamide; the compounds eluted at 9 and 7 min, respectively, at a flow rate of 1 mL min^{-1}. (*p*)-Nitroaniline, the hydrolytic product of benzyloxycarbonyl-Gly-Gly-Leu-*p*-nitroanilide, was detected using a UV/visible spectrophotometer at 405 nm.

Results

Initial hydrolysis rates were obtained from the first 10–20% of proteolysis. Proteolytic activity was expressed as the amount of substrate hydrolysed (g protein)$^{-1}$ min^{-1}. All epithelial cells studied had chymotrypsin-like, trypsin-like, and cucumsin-like activities in cytosol (Table 1). Effects of inhibitors on cytosolic degradation of insulin and IGF-I are summarized in Table 2. Less than 40% of insulin-degrading activities in alveolar cytosol was inhibited by aprotinin, a strong inhibitor of proteasome activity. Further, other inhibitors inhibited less than 20% of insulin-degrading activities in alveolar cytosol. In rectal cytosol, inhibition of IGF-I degradation by aprotinin, leupeptin, soybean trypsin inhibitor and chymostatin was similar, showing 25–30% inhibition. In Caco-2 cytosol, inhibition of IGF-I was less than 20% with the majority of inhibition less than 10%.

Table 1. Cytosolic proteolytic activities (μmol min (g protein)$^{-1}$) in epithelial cells.

Tissues	Trypsin-like	Chymotrypsin-like	Cucumsin-like
Caco-2 cells	110 (4·5)	1·2 (0·2)	190 (4·0)
Colon	13 (1·2)*	0·03 (0·001)*	6 (0·4)*
Rectum	7·1 (0·9)	0·5 (0·08)	21 (1)
Nasal	110 (9)	18 (1·5)	245 (8)
Alveolar	89 (2·9)	3·0 (0·5)	230 (8)

n = 6 for all except those marked *, for which n = 7.

Table 3. Inhibition of degradation of insulin rectal mucosal homogenate.

Inhibitors	% inhibition
Control	0
Aprotinin (0·5 mg mL^{-1})	0
Leupeptin (0·1 mg mL^{-1})	0
Diphosphofluoridate (1 mM)	0
Soybean trypsin inhibitor (0·1 mg mL^{-1})	0
Chymostatin (0·07 mg mL^{-1})	10 ± 0·5

Mean ± s.e.

Inhibition of insulin degradation by rectal homogenate is summarized in Table 3. Neither aprotinin, leupeptin, diphosphofluoridate nor soybean trypsin inhibitor inhibited insulin degradation by rectal homogenate. Only chymostatin achieved 10% inhibition.

Discussion

Although trypsin was used in the isolation of alveolar epithelial cells, the possibility of trypsin being absorbed by alveolar epithelial cells, resulting in the artifactual presence of trypsin-like activity in alveolar cytosol, is unlikely since trypsin is a large protein. Further, alveolar cytosol seemed to have the same order of chymotrypsin-like < trypsin-like < cucumsin-like activity as the majority of other epithelial cells. Colonic cytosolic activities had an order of trypsin-like > cucumsin-like > chymotrypsin-like activity. If a substantial amount of trypsin was absorbed into alveolar epithelial cells, its activity should have been much higher than that of the other two enzymes. Importantly, insulin degradation by alveolar cytosol was mainly due to cytosolic insulin-degrading enzyme, not trypsin-like activity (data not shown). Therefore, it is concluded that trypsin-like activity is present in the alveolar cytosol.

Although the above mentioned enzyme activities may show a certain pattern, it does not necessarily imply that in epithelial cells, proteins would encounter less chymotrypsin-like activity and more of the other two proteolytic activities. The order of activities is probably effected by specific substrates and their concentrations used, and by the kinetic parameters of the specific reactions between the enzyme and the substrates. Chymotrypsin-like activity preferentially cleaves the peptide bonds involving hydrophobic amino acid residues, trypsin-like activity degrades the peptide bonds involving basic amino acid residues, and cucumsin-like activity attacks the peptide bonds involving acid amino acid residues. Presumably, collective activities of these endopeptidases will be very powerful in eliminating intracellular proteins and in ensuring that all intracellular proteins are efficiently inactivated. Their role in degrading peptide and protein drugs by epithelial tissues should be understood in order to improve nonparenteral delivery of this class of therapeutic agents.

Aprotinin, leupeptin and chymostatin are strong inhibitors of proteasome activities, while diphosphofluoridate and soybean trypsin inhibitor are weak inhibitors of the multicatalytic enzymes (Arrigo et al 1988; Tsuji & Kurachi 1989). Therefore, the extent of inhibition by the former three inhibitors should reveal the role of proteasome-like activities in degrading insulin and IGF-I. The results of inhibition studies suggest that these proteolytic activities are not substantially involved in degradation of insulin and IGF-I by alveolar, rectal and Caco-2 cytosol. It has been observed that insulin degradation by Caco-2 cytosol is mainly due to insulin-degrading enzyme (data not shown). Therefore, it is concluded that degradation of insulin and IGF-I in epithelial tissues is not extensively due to the above three distinct proteolytic activities. However, it is unknown whether there are intracellular trafficking processes which are required to activate the involvement of proteasome, a newly-discovered multicatalytic complex, in intracellular degradation of peptide and protein drugs.

Table 2. Inhibition of cytosolic degradation of insulin-like growth factor I (IGF-I) and insulin.

Inhibitors	% inhibition of degradation of		
	Insulin (alveoli)*	IGF-I (rectum)*	IGF-I (Caco-2 cells)*
Control	0	0	0
Aprotinin (0·5 mg mL^{-1})	36 ± 2·9	29 ± 1	16 ± 0·9
Leupeptin (0·1 mg mL^{-1})	4·5 ± 0·3	32 ± 2·1	0
Diphosphofluoridate (1 mM)	4 ± 0·8	0	2·3 ± 0·5
Soybean trypsin inhibitor (0·1 mg mL^{-1})	13·7 ± 1·7	26 ± 2·3	3 ± 0·7
Chymostatin (0·07 mg mL^{-1})	6 ± 0·5	25 ± 1·7	6 ± 0·9

The data are the mean value of three experiments unless specified otherwise. Mean ± s.e. * The degradation of each protein hormone was by the cytosol prepared from the tissue indicated in parentheses.

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