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High levels of exopeptidase activity are present in rat and canine bronchoalveolar lavage fluid

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Summary

Although reduced loss from proteolytic activity is a potential advantage of the respiratory route for peptide and protein drug delivery, except for elastase and enkephalinase, few data are available regarding relevant enzyme levels. Protease activity in bronchoalveolar lavage (BAL) fluid from rat and dog has been examined to determine whether degradation is a potential limiting factor for peptides delivered via the lung in these animal models. Normal levels of endopeptidases cleaving substrates preferred by elastase, trypsin, and chymotrypsin are low in BAL fluid of both species, consistent with preliminary published data indicating that the stability of proteins may be much greater in the respiratory than the gastrointestinal tract. Aminopeptidase, dipeptidyl peptidase IV, and prolyl carboxypeptidase activity are found in BAL fluid: aminopeptidase levels are sufficiently high to be a potential limiting factor for peptide absorption after pulmonary administration in either species. In situ assays confirm that aminopeptidase activity detected in rat lavage fluid is only a fraction of that present at the surface of cells lining the respiratory tract. While low levels of active endoproteases probably do not pose a major enzymatic barrier, peptides susceptible to exopeptidases may require protection against degradation, e.g., by structural modification or protease inhibition, for optimal delivery via the respiratory tract.

Introduction

Since systemic absorption of peptide drugs administered via the gastrointestinal route is usually very poor, a great deal of attention is being focused on alternate routes of delivery. Administration via the respiratory tract has several potential advantages over oral delivery, including: (1) high volume and rate of blood supply; (2) less dilution of drug due to smaller volume of fluid within the respiratory tract; (3) simultaneous exposure of a large absorptive surface area (approx. 140 m^2) via aerosol delivery; (4) potentially higher effective permeability to peptides and proteins than the gastrointestinal epithelium; and (5) relatively low levels of protease and peptidase activity

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Abbreviations: BAL, bronchoalveolar lavage; PBS, Ca^{2+}-and Mg^{2+}-free phosphate-buffered saline; SUPHEPA, *N*-succinyl-L-phenylalanine-*p*-nitroanilide; SLAPN, *N*-succinyl-trialanyl-*p*-nitroanilide.

compared to the gastrointestinal tract (Patton and Platz, 1992). Although lack of proteolytic activity has often been assumed, data to support and quantitate this are extremely limited (see Discussion and Table 6. When examined in tissue homogenates, the lungs as a whole possess high levels of activity of a wide spectrum of hydrolytic enzymes (Crooks and Damani, 1989), including proteases (preferring high molecular weight substrates). However, little information on activity of exopeptidases (preferring low molecular weight substrates, or cleaving at or near the ends of larger molecules: McDonald, 1985; Lee, 1988) in the lung epithelium is available. In addition, the relevance of these observations to delivery of hydrophilic peptides is unclear, since a hydrophilic drug molecule introduced into the respiratory tract would not be expected to encounter intracellular enzymes, but only those present at cell surfaces or released into the extracellular milieu. Hydrophilic peptide transport is likely to involve paracellular and possibly transcytotic routes, neither of which expose a drug to cytoplasmic or lysosomal enzymes.

We have measured levels of several proteolytic enzymes in bronchoalveolar lavage fluid from rats and dogs to estimate the degradative liability which might be encountered by peptides or proteins delivered by this route. Levels of exopeptidase activity detected are sufficiently high for some enzymes to indicate that protection of peptides against degradation by structural modification or addition of enzyme inhibitors may be necessary to optimize their absorption via this route.

Materials and Methods

Materials

N-Succinyl-trialanyl-*p*-nitroanilide (SLAPN), aprotinin, *N*-succinyl-L-phenylalanine-*p*-nitroanilide (SUPHEPA), leucine-*p*-nitroanilide, hippuryl-L-phenylalanine (*N*-benzoyl-Gly-Phe), elastase (porcine pancreatic type 4), leucine aminopeptidase (type III-CP), L-amino acid oxidase, horseradish peroxidase (type IV), 1,10-phenanthroline, bestatin, and *o*-dianisidine were from Sigma Chemical Co., St. Louis, MO. Chromozym® TRY (carbobenzoxy-valyl-glycyl-arginine-4-nitroanilide acetate), chymotrypsin A_4, trypsin, and carboxypeptidase A (from bovine pancreas), were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). The substrates Gly-Pro-pNA · Tos (GPNT), Z-Gly-Pro-Leu, Glu-pNA, and Ala-pNA were purchased from Peninsula Laboratories, Inc. (Belmont, CA). All other chemicals used were reagent grade.

Rats were male Sprague-Dawley (strain CD, 250–700 g) obtained from Charles River (Boston, MA). Dog lavage fluid was obtained from beagles.

Collection of bronchoalveolar lavage fluid

Rats were anesthetized with nembutal, i.p., 80 mg/kg body weight. Skin was reflected by a straight central cut from the intestine to the chin, the abdomen opened, and the rat bled by severing the inferior vena cava. The chest cavity was opened from the anterior end of the rib cage to expose the lungs. Muscle layers were removed from the trachea, 5–10 ml (depending on the size of the animal) of PBS, pH 7.4 was injected into the trachea, and immediately aspirated slowly. The lavage procedure was repeated once with the same volume of PBS, and the aspirates pooled in 50 ml conical tubes on ice. Lavage fluid was centrifuged for 10 min at $500 \times g$ (4°C) to separate cells from fluid. The low speed pellet of cells was resuspended by vortex mixing after addition of 1 ml of PBS. Samples were frozen in aliquots at −80°C or further subfractionated (see below) prior to assay. In some cases, 0.9% NaCl was used instead of PBS for lavage, since the presence of phosphate in PBS resulted in formation of precipitates during assay of glutamyl aminopeptidase activity, which requires Ca^{2+} for activity.

Beagle dogs were euthanised with sodium pentobarbital, and intubated prior to lung lavage with 60 ml PBS or normal saline (0.9% NaCl) each wash, 3–4 washes per animal. Removal of cells was performed as described for rats.

Any lavage samples containing blood were discarded.

Fractionation of lavage fluid

Supernates obtained from the $500 \times g$ centrifugation were further subfractionated into soluble and particulate (membrane vesicle) fractions by sedimentation at 45 000 rpm ($100\,000 \times g$) for 90 min at 4°C in the 80Ti rotor (Beckman Instruments, Palo Alto, CA). High speed pellets obtained from this procedure were resuspended in 1 ml of PBS, using a 2 ml Dounce homogenizer (Kontes Glass Co., Vineland, NJ). Samples were stored at −80°C until use.

Enzyme assay procedures

Elastolytic activity Elastase activity was measured with the synthetic substrate succinyl-L-tri-alanine-nitroanilide (SLAPN) by the method of Valentine and Fisher (1984). 50–200 μl of lavage fluid was incubated with 10 mM substrate in 0.2 M Tris-HCl, pH 8, 5 mM $CaCl_2$ with continuous shaking at 37°C. Hydrolysis of substrate to produce *p*-nitroaniline was monitored spectrophotometrically at 410 nm over a 24–48 h incubation period. Product concentration was determined from a standard curve of *p*-nitroaniline concentration vs absorbance. Formation of reaction product continued to increase in a linear manner for as long as 72 h.

Trypsin-like activity Activity was measured against the substrate Chromozym® TRY, according to the protocol supplied with the substrate. 200–500 μl of lavage fluid was brought to 1 ml volume with PBS, and reactions started by addition of 83 μl of 10 mM substrate. Hydrolysis of substrate into carbobenzoxy-valyl-glycyl-arginine and *p*-nitroaniline was monitored by the increase in absorbance at 410 nm after 24–48 h incubation with shaking at 37°C. Formation of reaction product was linear over this time period.

Chymotrypsin-like activity Activity was measured against the substrate SUPHEPA, which was hydrolyzed into *p*-nitroaniline and succinyl-L-phenylalanine. 200–500 μl of lavage fluid was brought to 1 ml volume by addition of PBS, and reactions started by addition of 33.4 μl 50 mg/ml SUPHEPA in dimethylformamide. Reactions were incubated for 24–48 h at 37°C in a shaking water bath, and formation of product followed by monitoring the absorbance at 410 nm. Formation of reaction product was linear over this time period.

Aminopeptidase activity Leucine aminopeptidase activity was measured by following the release of *p*-nitroaniline from leucine-*p*-nitroanilide (Roman and Hubbard, 1983). 10–200 μl aliquots of lavage fluid or fractions obtained from them by differential centrifugation were brought to 0.35 ml by addition of PBS, 50 μl of 0.5 M Na phosphate, pH 8 was added, and the reactions started by addition of 100 μl 20 mM substrate in water. After 1 h at 37°C in a shaking water bath, reactions were stopped by addition of 50 μl 40% trichloroacetic acid. Absorbance at 410 nm was determined after addition of 0.45 ml alkaline reagent (133 mM glycine, 83 mM Na_2CO_3, 67 mM NaCl adjusted to pH 10.7 with NaOH).

Comparison of activity against leucine-, alanine-, and glutamic acid *p*-nitroaniline was determined by a protocol similar to that described above, using either 50 μl 0.5 M Na phosphate, pH 7 (alanine or leucine) or 250 μl 0.2 M Tris-HCl, pH 7 and 10 μl 1 M $CaCl_2$ (glutamic acid) as the buffer in place of the 50 μl Na phosphate, pH 8 used in the LAP assay. The concentration of all substrates in the comparative assays was 1.5 mM, in a final volume was adjusted to 0.5 ml by addition of the appropriate volume of water.

Assay of rat lung aminopeptidase activity in situ was performed by instillation of 5 ml of PBS containing 5.76 mg of leucine-*p*-nitroanilide into the lungs by the normal lavage procedure, waiting 1–3 min, then aspirating the lavage fluid. Fluid was collected on ice, and reactions stopped by addition of proportional amounts of trichloroacetic acid and alkaline reagent, as described above. Cells were removed by centrifugation, and absorbance of the supernatant fluid read at 410 nm.

Inhibition by 10 mM 1,10-phenanthroline or by 0.01 mM bestatin was assessed by addition of the inhibitors directly to the assay mixture.

Prolyl carboxypeptidase activity Prolyl carboxypeptidase A activity was measured by monitoring the production of free L-leucine during hydrolysis of 1 mM Z-Gly-Pro-Leu in 50 mM Tris-HCl, pH 8.0 (Nicholson and Kim, 1975; Erickson, et al., 1989). Samples were incubated

for 1 h at 37°C in a shaking water bath with L-amino acid oxidase reagent (Nicholson and Kim, 1975), and reactions terminated by addition of 0.74 ml 50% sulfuric acid before reading absorbance at 530 nm.

Dipeptidyl peptidase IV activity Dipeptidase activity was measured with the substrate Gly-Pro-pNA · Tos, using the procedure of Nagatsu, et al. (1976), with glycine-NaOH buffer, pH 8.7. After 60 min incubation with shaking at 37°C, *p*-nitroaniline was detected by addition of trichloroacetic acid and alkaline reagent, as described for assay of leucine aminopeptidase.

Protein determinations Protein was measured by the method of Bradford (1976), using BSA as the protein standard and dye reagent mix obtained from BioRad Laboratories (Richmond, CA).

Alkaline phosphodiesterase This plasma membrane marker enzyme was assayed as described by Hubbard et al. (1983).

Results

Measurement of activity of three endopeptidases using substrates cleaved by either elastase, trypsin, or chymotrypsin revealed either low or undetectable levels in rat (Table 1) and dog (Table 3) BAL fluid. These determinations were performed with low speed supernatant fluid obtained after removal of whole cells by centrifugation, and therefore do not include activity which may be associated with the cells themselves (predominantly macrophages). Measurement of cell-associated activity of these enzymes was not performed, since they are soluble enzymes which would not be expected at the cell surface, and a hydrophilic drug molecule would not be likely to encounter intracellular enzymes.

In contrast to low levels of endopeptidase activity, substantial amounts of exopeptidases were detected in rat and dog BAL (Tables 1 and 3), using substrates cleaved by either aminopeptidases (leucine-, alanine-, or glutamic acid-*p*-nitroanilide), or dipeptidyl peptidase IV (Gly-Pro-pNA · Tos). Prolyl carboxypeptidase activity (using Z-Gly-Pro-Leu as substrate) was also detected in lesser amounts. Since the amount of fluid instilled was sufficient to fill only approximately half of the available volume of the dog lungs, values for total enzyme activity are underestimated.

TABLE 1

Protease activity of rat BAL fluid

Enzyme	Total activity [a]	Specific activity [b]	*n*
Elastase	365 ± 39	0.094 ± 0.014	3
Trypsin	50 ± 11	0.013 ± 0.003	3
Chymotrypsin	n.d.	n.d.	2
Aminopeptidase [c]	5745 ± 636	1.65 ± 0.39	3
Prolyl carboxypeptidase	122 ± 9	0.056 ± 0.013	4
Dipeptidyl peptidase IV	1376 ± 739	0.67 ± 0.39	4

[a] nmol/h total activity in BAL fluid.

[b] nmol substrate hydrolyzed/h per μg protein at 37°C.

[c] Activity measured with leucine-*p*-nitroanilide substrate, pH 8.

n.d., not detectable; *n*, number of animals from which samples were obtained. Activities are mean ± standard deviation of values for *n* animals.

Since both soluble and membrane-associated forms of many exopeptidases have been described (see Discussion), we investigated whether aminopeptidase activity seen in BAL was associated with soluble or particulate fractions after differential centrifugation, using leucine-*p*-nitroanilide as substrate (Fig. 1). 77% of total aminopeptidase activity recovered in rat BAL was found with either the low speed cell pellet or with the high speed pellet (Table 2 calculated as total activity recovered in both pellets with correction for the % of low speed supernatant fraction used for the high speed spin). Canine lavage fluid gave similar results, although a somewhat greater amount of activity remained in the supernatant fraction after high speed centrifugation. (48 ± 11% of total recovered activity with both pellets: Table 4). The high speed pellet is likely to include membrane fragments derived from epithelial cells and/or macrophages of the respiratory tract, an expectation supported by detection of activity of a plasma membrane marker enzyme, alkaline phosphodiesterase, in the high speed pellet, but not in the

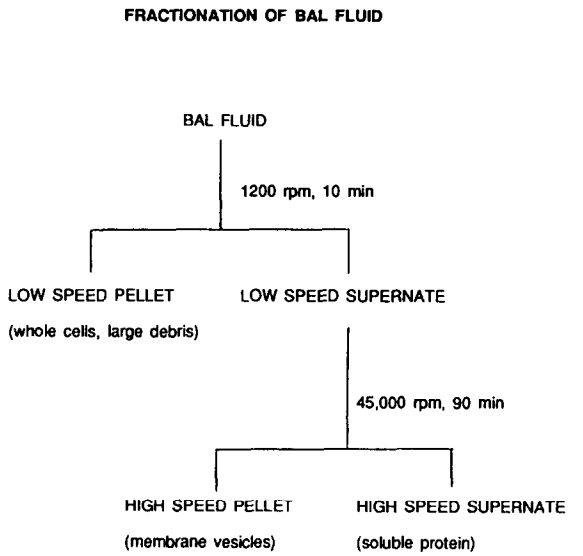

Fig. 1. Protocol for differential centrifugation of BAL samples.

high speed supernatant fraction from rat and dog (3 nmol substrate hydrolyzed/mg protein per h by rat high speed pellet; 0.09 nmol substrate hydrolyzed/mg protein per h by dog).

TABLE 2

Aminopeptidase activity of rat BAL fractions

Fraction	Expt	Total activity [a]	Specific activity [b]
Low speed supernate	1	5093 ± 489	1.43
	2	6364 ± 431	1.41
Low speed pellet	1	833 ± 51	1.62
High speed supernate [c]	1	320 ± 41	0.38
	2	476 ± 14	0.57
High speed pellet [c]	1	882 ± 38	5.69
	2	886 ± 60	7.2

[a] nmol substrate hydrolyzed/h at 37°C: values are mean ± standard deviation.

[b] Average nmol substrate hydrolyzed/h at 37°C/average μg protein for that sample.

[c] 4 ml out of 15 ml total volume of low speed supernate used for high speed spin in Expt 1; 3.5 ml out of 15 ml total volume of low speed supernate used for high speed spin in Expt 2.

Aminopeptidase activity measured against leucine-*p*-nitroanilide at pH 8.

TABLE 3

Protease activity of dog BAL fluid

Enzyme	Total activity [a]	Specific activity [b]	*n*
Elastase	334 ± 137	0.009 ± 0.003	4
Trypsin	63 ± 24	0.0013 ± 0.0004	3
Chymotrypsin	12 ± 3	0.0004 ± 0.0004	3
Aminopeptidase [c]	10319 ± 7670	0.46 ± 0.08	3
Prolyl carboxypeptidase	376 ± 373 [d]	0.012 ± 0.003	3
Dipeptidyl peptidase IV	1237 ± 537	0.083 ± 0.07	2

[a] nmol/h total activity in BAL fluid: total lung volume not filled during lavage.

[b] nmol substrate hydrolyzed/h per μg protein at 37°C: values are mean ± standard deviation.

[c] Activity measured against leucine-*p*-nitroanilide at pH 8.

[d] Large variation due to much higher values obtained in samples from one animal: 147, 173 and 807 total nmol/h recovered in three individuals.

n, number of animals from which samples were obtained.

For all three exopeptidases, 6–21% of recovered activity in lavage fluid was found in the low speed pellet, which consists largely of alveolar macrophages, indicating that these enzymes are expressed by at least one of the cell types normally found within the alveolar lumen.

The possibility that a large amount of exopeptidase activity is associated with pulmonary epithelial cells and was therefore underestimated by determining activity in lavage fluid was investigated by instilling the aminopeptidase assay medium directly into the lungs of rats. Aminopeptidase was chosen for this approach because enzyme activity could be determined by adding leucine-*p*-nitroanilide directly to isotonic PBS used as the lavage medium. The results indicated total enzyme levels capable of cleaving 66 ± 31 μmol of leucine-*p*-nitroanilide/h ($n = 3$), substantially higher than levels estimated from assay of lavage fluid (5.7 μmol/h total activity: Table 1).

The nature of the aminopeptidase activity of lavage fluid was further investigated by comparing enzyme activity against three different substrates: leucine-, alanine-, and glutamic acid-*p*-nitroanilide (Table 5). Both low speed super-

natant and pellet fractions from rat hydrolyzed the alanyl substrate more rapidly than the leucyl or glutamic acid analogues. These data suggest that the enzyme(s) predominantly responsible for the measured activity is not leucine aminopeptidase, and likely includes other enzymes, such as aminopeptidase A and/or N (see Discussion). In dog samples, both leucine- and alanine-*p*-nitroanilide were cleaved at similar rates by low speed supernatant fractions, while aminopeptidase activity detected in the cell pellet preferred the leucine analogue. Glutamic acid substrates were relatively poorly hydrolyzed by both fractions in samples obtained from one animal lavaged with saline instead of PBS.

TABLE 4

Aminopeptidase activity of dog BAL fractions

Fraction	Expt	Total activity [a]	Specific activity [b]
Low speed supernate	1A	7298 ± 706	0.57
	1B	2558 ± 120	0.45
	2A	10028 ± 1146	0.38
	2B	20678 ± 3332	0.45
	3	13167 ± 1333	0.52
Low speed pellet	1A	3329 ± 976	0.47
	1B	428 ± 117	0.98
	2A	2329 ± 482	5.3
	2B	4428 ± 804	9.1
	3	3841 ± 251	4.8
High speed supernate [c]	1A	615 ± 32	0.32
	1B	547 ± 72	0.25
	2A	2367 ± 360	0.40
	2B	3015 ± 1457	0.53
	3	1614 ± 162	0.34
High speed pellet [c]	1A	270 ± 29	0.83
	1B	144 ± 28	0.88
	2A	1332 ± 353	1.93
	2B	3365 ± 688	3.3
	3	853 ± 136	1.7

Experiment numbers refer to three different dogs, letters to different lavages from an animal.

[a] nmol substrate hydrolyzed/h at 37°C: values are mean ± standard deviation.

[b] Average nmol substrate hydrolyzed/h at 37°C/average μg protein for that sample.

[c] 5 ml out of 38 ml total volume of low speed supernate used for high speed spin in 1A; 5 ml of 17 ml in 1B; 10 ml of 29 ml in 2A, 10 ml of 45 ml in 2B, 10 ml of 44 ml in 3.

Aminopeptidase activity measured with leucine-*p*-nitroanilide substrate at pH 8.

TABLE 5

Comparison of aminopeptidase activity against different substrates

	Sample	Substrate	Relative activity	No. of animals used
Rat	LS SPT	Ala	1.6	$n = 6$ for Ala, Leu
		Leu	1.0	$n = 3$ for Glu
		Glu	1.1	
	LS PEL	Ala	1.33	$n = 1$
		Leu	1.0	
		Glu	0.73	
Dog	LS SPT	Ala	1.1	$n = 2$ for Ala, Leu
		Leu	1.0	$n = 1$ for Glu
		Glu	0.15	
	LS PEL	Ala	0.5	$n = 2$ for Ala, Leu
		Leu	1.0	$n = 1$ for Glu
		Glu	0.2	

Activity in different samples was normalized by setting the amount of leucine *p*-nitroanilide hydrolyzed to a value of 1, and expressing activity against the other substrates relative to this level. LS, low speed; HS, high speed; SPT, supernate; PEL, pellet.

Inclusion of 1,10-phenanthroline (reported to be an inhibitor of aminopeptidase A and N activity in renal tissue: Jackson et al., 1988) in the assay medium resulted in a variable extent of inhibition ranging from 15 ± 2 to $78 \pm 1\%$ ($n = 3$ animals) reduction in the level of activity detected in rat low speed supernates using leucine-*p*-nitroanilide as substrate. The same compound had no inhibitory effect ($n = 2$ animals) on activity of dog BAL low speed supernates. In analagous assays with the same substrate, 0.01 mM bestatin (reported to inhibit leucine aminopeptidase, aminopeptidase N, and aminopeptidase B to varying extents: Stratford and Lee, 1986) caused inhibition ranging from 20 ± 5 to $35 \pm 8\%$ inhibition ($n = 3$ animals) of rat low speed supernate aminopeptidase activity, but only $11 \pm 4\%$ inhibition of activity in the lavage supernate from a dog. Increasing the level of bestatin to 0.025 mM had no effect on the level of inhibition seen in samples from one rat.

Discussion

The data presented here demonstrate similar profiles of activity for several exo- and endopeptidases in lavage fluid from rat and dog. In both species, levels of endopeptidases detected are low, while aminopeptidase activity is high enough to be of potential significance for pulmonary delivery of peptide drugs.

Low levels of elastase activity are expected in BAL fluid, and indicate that the lavage procedure used provides an accurate sample of airway and alveolar content. Elastase and collagenase activity in BAL supernates from normal, non-smoking humans ranges from very low to undetectable, although levels are elevated in some cases of lung pathology (Gadek et al., 1979; Hayem et al., 1980; Lee et al., 1981; Janoff et al., 1983; Smith et al., 1985; Fujita et al., 1990). Little information has been published on BAL fluid proteases or peptidases other than elastase (Table 6). In one report, high levels of chymotrypsin-like activity (300–600 μmol/h total activity in an unspecified volume of lavage fluid) were detected in human lavage fluid from normal, non-smoking individuals (Hayem et al., 1980). This contrasts with very low to undetectable levels of this enzyme found in both rat and dog BAL in our work, and may result from differences in substrate used, concentration of enzyme activity in fluid collected, species variation between humans and the animals used, or other factors. Prolyl endopepti-

TABLE 6

Summary of published reports of protease activity potentially relevant to pulmonary peptide delivery

Enzyme	Species	Detection method	Location [a]	Reference
Endoproteases				
Elastase, collagenase	human	biochemical assay	lavage fluid	many reports [b]
Chymotrypsin activity	human	biochemical assay	lavage fluid	Hayem et al. (1980)
Prolyl endopeptidase	human	biochemical assay	lavage fluid	Orlowski et al. (1981)
Cathepsin B activity	human	biochemical assay	lavage fluid	Orlowski et al. (1981)
Enkephalinase	human	cytochemistry, biochemical assay	tracheal, alveolar epithelium	Johnson et al. (1985), Borson (1991)
Exopeptidases				
Glutamyl aminopeptidase [c]	rat	cytochemistry	epithelium, endothelium	Gossrau (1985)
γ-Glutamyl transpeptidase	rat	cytochemistry	bronchial epithelium	Gossrau (1985)
Dipeptidyl peptidase IV	rat, rabbit	cytochemistry	endothelium, macrophages	Gossrau (1985), Sannes (1983), Křepela et al. (1985)
Alanyl aminopeptidase [d]	rat	cytochemistry	macrophages	Gossrau (1985)
Angiotensin converting enzyme	human, rat, pig	immunocytochemistry, biochemical assay	endothelium	Johnson et al. (1985), Ryan et al. (1976)
Arginine carboxypeptidase	bovine	immunocytochemistry, biochemical assay	endothelium	Ryan and Ryan (1983)

[a] Unless specified, cell types listed refer to alveolar epithelium, capillary endothelium, or alveolar macrophages.

[b] See Discussion for literature citations: only reports providing information regarding cell type with which enzymes are associated are included.

[c] Also known as aminopeptidase A.

[d] Also known as aminopeptidase N or M.

dase and a cathepsin B-like activity in human, rat and rabbit BAL fluid were associated predominantly with a low speed pellet containing alveolar macrophages (Orlowski et al., 1981). Both absolute amounts and the ratio of cell-associated vs supernatant fluid activity were highly variable for both enzymes in human samples from different individuals in a clinically normal population. Low levels of protease activity could result from low amounts of the enzymes or from the presence of inhibitors such as α_1-antitrypsin and antichymotrypsin in alveoli (Hayem et al., 1980; Janoff, 1985).

Distribution of membrane proteases in lung tissue has been examined cytochemically by Gossrau (1985: summarized in Table 6). Although cytochemistry does not allow exact quantitation of levels of enzyme activity, and the specificity of substrates for a single enzyme has been questioned (e.g., Patterson et al., 1963; Sylven and Bois, 1963), these results suggest that a variety of exopeptidases may be encountered by peptide drugs administered via the respiratory tract, supporting the quantitative data presented in this report.

Biochemical and immunochemical analyses have demonstrated that dipeptidyl aminopeptidase IV, microsomal alanyl aminopeptidase, and glutamyl aminopeptidase are predominantly associated with brush border membrane fractions of small intestine and renal tubules (Miura et al., 1983; Semenza, 1986). Among the brush border membrane-associated enzymes, glutamyl aminopeptidase (aminopeptidase A: Benajiba and Maroux, 1980; Gorvel et al., 1980), and microsomal alanyl aminopeptidase (aminopeptidase N: Benajiba and Maroux, 1980; Ahnen et al., 1982), are both capable of cleaving either leucine β-napthylamide or leucine-*p*-nitroanilide at significant rates, in addition to the alanyl- and glutamyl-substrates which they prefer.

Our fractionation data demonstrate that a large proportion of aminopeptidase activity in rat BAL is associated with the low speed cell pellet or with particles sedimenting only at high centrifugal force. The enzyme(s) responsible for this activity are therefore likely to be associated with membrane vesicles derived from plasma membranes of either macrophages or epithelial cells lining the airways or alveoli. For this reason, most aminopeptidase activity detected is not likely due to leucyl aminopeptidase (EC 3.4.11.1), a cytosolic enzyme that is soluble, not membrane-associated. Further information regarding the identity of the enzyme(s) responsible for aminopeptidase activity is obtained from comparison of activity against leucine-, alanine-, and glutamic acid-*p*-nitroanilides. The relatively high rate of alanyl substrate cleavage indicates that BAL activity is due primarily to aminopeptidase A and/or N, and possibly other enzymes as well (Gorvel et al., 1980; Stratford and Lee, 1986). Finally, lack of complete inhibition of rat aminopeptidase activity caused by 1,10-phenanthroline or bestatin provides a further argument for the presence of several different aminopeptidases (assuming that enzymes from different tissues have similar sensitivity to this inhibitor: Jackson et al., 1988). Comparison of substrate specificity of aminopeptidases in rabbit nasal homogenates also provides evidence for the presence of multiple aminopeptidases in the upper respiratory tract (Stafford and Lee, 1986).

In the gastrointestinal tract, most pancreatic proteases are endopeptidases which prefer to cleave internal bonds of relatively large polypeptides (Lee, 1988; Lee and Yamamoto, 1990). Small peptides are usually relatively resistant to pancreatic protease hydrolysis, and are more susceptible to cleavage by brush border (apical plasma membrane) and cytosolic peptidases (Adibi, 1971). In the respiratory tract, soluble endopeptidase levels appear relatively low in the alveolar lumen. However, exposure of inhaled drugs to the large surface area of the lung to achieve rapid absorption could also result in rapid degradation by cell surface enzymes. Hydrolysis of a wide variety of peptides has been attributed to neutral endopeptidase (enkephalinase), a cell-surface enzyme associated with airway epithelium and alveolar lining cells (Johnson, et al., 1985; Borson, 1991). In addition, the data presented here demonstrate high lung exopeptidase levels. The extent of aminopeptidase liability of peptides delivered to the respiratory tract can be estimated from our rat experiments (see Appendix for details). Direct

instillation of leucine-*p*-nitroanilide into rat lungs demonstrated sufficient levels of enzyme activity to cleave approx. 1 mg of an unprotected 1000 Da peptide within 1 min. Although rates of peptide hydrolysis may be different than that of the assay substrate, there is no reliable way to estimate this, since natural substrates for the enzymes assayed may be cleaved at either faster or slower rates (e.g., aminopeptidases A and N from rabbit and pig intestine have higher turnover numbers for tripeptide substrates than for amino acid-*p*-nitroanilides: Feracci et al., 1981). In addition, neither the lavage nor instillation procedure will reveal activity associated with the surface of capillary endothelial cells (Table 6), which also would be encountered by absorbed peptides.

Although our data only provide an estimate of the potential protease liability associated with respiratory delivery, the results indicate that protection of susceptible peptides against degradation by structural design or other means may be required for optimal delivery by the respiratory route. Comparison of the levels of enzyme activity in human lavage fluid with those detected in material from animal models will be necessary to determine the relevance of these findings for clinical studies. High levels of absorption of several peptides structurally designed for resistance to degradation are seen after delivery to the lung in animal models and humans (e.g., leuprolide acetate: Adjei and Garren, 1990; Adjei et al., 1990; and 1-deamino-cysteine-8-D-arginine vasopressin: Folkesson et al., 1990). A significant level of pulmonary absorption of both insulin and growth hormone has also been seen, although some degradation of growth hormone occurred, and the effect of addition of protease inhibitors on absorption of these structurally unprotected molecules was not investigated (Elliott et al., 1987; Patton et al., 1990; Colthorpe, et al., 1992; Laube et al., 1992). Possible complications which could result from inhibition of enzymes involved in normal lung physiology, for example, in regulating turnover rates of peptide mediators of airway function (Borson, 1991; Caughey, 1992), would need to be evaluated before addition of inhibitors could be recommended for formulation of proteins for pulmonary delivery.

Appendix: Calculation of Potential Aminopeptidase Liability for a Hypothetical Peptide Delivered to Rats via the Pulmonary Route

Assumptions

- Molecular mass = 1000 Da for hypothetical peptide;
- Aerosol delivery exposes a similar % of the total epithelial surface area as substrate instillation;
- Rate of peptide hydrolysis is similar to leucine-*p*-nitroanilide.

Total aminopeptidase activity measured from instillation of substrate directly into the lung = 66 μmol substrate hydrolyzed per h.

$$66\ \mu\text{mol/h} \times 1000\ \mu\text{g/}\mu\text{mol}$$

$$= 66\ \text{mg/h} = 1.1\ \text{mg/min of peptide hydrolyzed}$$

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