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International Journal of Pharmaceutics 195 (2000) 93–101

international journal of pharmaceutics

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Characterization of proteolytic activities of pulmonary alveolar epithelium

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Received 9 September 1999; received in revised form 25 October 1999; accepted 26 October 1999

Abstract

Pulmonary alveolar type I epithelial cell and its progenitor, type II cell, present major transport and enzyme barriers for systemic delivery of pulmonary administered peptide drugs. The present study investigates the effect of cellular differentiation of type II to type I cells on their proteolytic activities, and evaluates the suitability of a continuous lung cell line, A549, for drug transport and degradation studies. High performance liquid chromatography was used to assess the degradation kinetics of two model peptide substrates, luteinizing hormone releasing hormone (LHRH) and [D-Ala^6]-LHRH, and their metabolites in lung cell preparations. Isolated primary type II cells when grown in culture developed tight monolayers and exhibited morphologic characteristics of type I cells, as determined by transepithelial electrical resistance measurements and electron microscopy. The transformed type I-like cells exhibited a >10-fold decrease in proteolytic activities for LHRH, as compared to type II cells. The continuous lung cell line A549 formed leaky monolayers and exhibited similar enzyme activities to the primary type II cells. The responsible enzymes for degradation of LHRH in type II and A549 cells were angiotensin converting enzyme (ACE), EP24.11, and EP24.15. In contrast, no EP24.15 or ACE activity was observed in type I-like pneumocytes and only a weak EP24.11 activity was detected. In all cell types, the degradation rate of [D-Ala^6]-LHRH was about 3–8 times lower than that of LHRH. This peptide analog was resistant to degradation by EP24.15 and EP24.11, but was susceptible to ACE-mediated cleavage.

Keywords: Peptide degradation; Lung; Enzyme activity; LHRH; A549; Epithelial cells

1. Introduction

The lung has increasingly been investigated as a site for systemic delivery of drugs. Drugs that are highly susceptible to first pass metabolism or undergo extensive GI degradation, e.g. peptides and proteins, have been demonstrated to exhibit an improved systemic bioavailability when administered through the lungs (see Patton and Platz, 1992; Byron and Patton, 1994 for reviews). While the pulmonary route of administration can deliver therapeutic quantities of peptides and proteins

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0378-5173/00/$ - see front matter
PII: S0378-5173(99)00377-4

into circulation, significant portions of their administered doses are generally lost as a result of enzymatic degradation by lung peptidases. A variety of lung enzymes have been identified and their role in peptide degradation has been investigated (Johnson et al. 1985; Choi et al., 1990; Wang et al., 1993). However, most studies to date have been conducted using intact lungs or whole lung homogenates, and therefore information about the mechanisms and specific enzyme activities in different lung cell types is not known. Recently, isolated lung cell systems have been developed and used to evaluate the enzyme and transport properties of lung pneumocytes (see Kim and Crandall, 1996 for review). Nonetheless, no studies have investigated the proteolytic activities of different lung cell types and the effect of cellular differentiation on their activities.

In the lung, the pulmonary alveolar region represents the major absorptive site for drugs. The total surface area of the alveoli in humans is >100 m^2 (Stone et al., 1992) and occupies $>90\%$ of the total lung surface (Scothorne, 1987). The alveolar surface is populated by two major epithelial cell types, the terminally differentiated type I cell and its progenitor type II cell (Adamson and Bowden, 1975; Bowden, 1981). The alveolar epithelium forms tight junction barrier which limits the transport of most drugs. In order to enter the systemic circulation, peptide and protein drugs must cross this barrier, while being subjected to enzymatic degradation. The nature and extent of proteolytic enzymes in type I and II cells have not been systematically investigated. In this study, we hypothesize that these cells present a significant enzymatic barrier for peptide absorption and that alveolar type I cells, because of their late state of differentiation, are metabolically less active and therefore possess lower proteolytic activities than the type II cells. To test this hypothesis, we isolated type II cells from rats and incubated them with LHRH and [D-Ala^6]-LHRH. LHRH is a peptide hormone which regulates the secretion of luteinizing hormone and follicle stimulating hormone (Shally et al., 1971). LHRH and [D-Ala*6*]-LHRH are commonly used as female contraceptives and for the treatments of infertility, hormone-sensitive prostate cancer, and premenopausal breast cancer (Fink et al., 1974; Okada et al., 1983). Because of their therapeutic importance and their well-defined degradative products, these compounds were chosen in this study. The degradation kinetics of the peptides in type II cells were determined and then compared with those of the type I cells. Because of the lack of appropriate methods to isolate and purify type I cells, the present study utilized primary culture of type II cells. These cells when grown in culture on microporous membrane supports formed tight monolayers and exhibited morphological and functional characteristics of type I cells. This cell culture method allows continuous assessment of the effect of cell differentiation on proteolytic activities, which could not be achieved in vivo.

Another important aspect of this investigation is the assessment of enzymatic and transport properties of alveolar lung cell line A549. While the primary culture of epithelial pneumocytes has proven useful for the studies of drug metabolism and transport, this method of investigation is technically difficult and time consuming. Alternatively, continuous lung cell lines such as the A549 can possibly be used to aid the studies. The A549 cell line has been used to investigate the absorption characteristics of various compounds including dextrans (Fujita et al., 1993), peptides and proteins (Kobayashi et al., 1995). However, the appropriateness of this cell culture system as a model for transport and degradation studies has been questioned and is therefore further investigated in this study.

2. Materials and methods

2.1. Materials

Male specific pathogen-free Sprague-Dawley rats weighing between 150–200 g (Hilltop Labs, Scottsdale, PA) were used. The alveolar epithelial A549 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). LHRH (LHRH 1–10), its analog [D-Ala^6]-LHRH, and various LHRH fragment standards (LHRH 7–10, LHRH 4–10, LHRH 3–10, LHRH 2–10) were purchased from Sigma (St

Louis, MO). LHRH 6–10 fragment was custom synthesized by Quality Control Biochemical (Hopkinton, MA). All peptides were HPLC purified and were $>90\%$ pure. All other reagents including captopril, thiorphan, and disodium ethylene diaminetetraacetic acid (EDTA) were purchased from Sigma.

2.2. Preparation of epithelial pneumocytes

Type II pneumocytes were harvested from rats by enzymatic digestion according to the method previously described (Castranova et al., 1988; Wang et al., 1993). The animals were anesthetized by intraperitoneal injection of sodium pentobarbital solution (0.2 g kg^{-1}). The trachea was cannulated and the lungs were lavaged with phosphate-buffered saline (PBS) to remove mobile cells. The lungs were then excised and filled with PBS containing elastase (40 units ml^{-1}, type I; U.S. Biochemical, Cleveland, OH) and deoxyribonuclease (DNase, 0.006%; Sigma) and incubated at 37°C for 20 min to free lung cells. After enzymatic digestion, the lungs were finely minced and the digestion was arrested by incubation for 5 min in PBS containing 25% fetal bovine serum and 0.006% DNase. The crude extract was sequentially filtered through 160- and 45-μm screens and centrifuged. The resulting cell pellet was spun on a sterile Percoll density gradient. The second cell band from the surface was collected, washed twice, and resuspended in 1:1 F12 and Eagle's modified essential medium, supplemented with 100 units ml^{-1} penicillin and 0.1 μg ml^{-1} streptomycin. The cell suspension yielded 5×10^6 cells/rat with viability $>95\%$ as determined by the Coulter counter (model AB, Coulter Instrument, Hialeah, FL) and trypan blue dye exclusion. The purity of the type II cell suspension, estimated by phosphine 3R fluorescent staining, was $>90\%$.

Type I-like pneumocytes were prepared according to the method previously described (Cheek et al., 1989; Wang et al., 1993). Briefly, freshly isolated type II cells from above were plated onto 0.4-μm-pore, 0.3-cm^2 tissue culture-treated polycarbonate filters (Costar, Cambridge, MA) at 1×10^6 cells per cm^2 on 24-well plates. The cells on filters were maintained in a humidified 5% CO_2 incubator at 37°C and the nutrient medium was changed every 48 h after plating. Transformation of cultured type II cells was monitored by electrical resistance measurements using the Millicell ERS testing device (Millipore, Bedford, MA) and by scanning electron microscopy. Data obtained from separate experiments indicated that the cuboidal type II cells transformed into a squamous, type I-like, monolayer after 6 days in culture. The transepithelial electrical resistance of this monolayer was $\approx 2000\ \Omega\ cm^2$ from day 6 to 10, after which it was gradually declined (see the Section 3 for more details). These morphologic and functional changes are consistent with those observed in developing type I cells in vivo (Cheek et al., 1989). Previous immunologic studies also showed that type II cells grown in culture developed a phenotypic characteristic of type I cells (Danto et al., 1992).

2.3. Cell line

The A549 cell line was maintained in F-12 K medium supplemented with 10% fetal bovine serum, 100 units ml^{-1} penicillin, and 0.1 μg ml^{-1} streptomycin at 37°C in a humidified atmosphere at 5% CO_2. Prior to use, the cells were washed and resuspended in PBS buffer. In some studies, the cells were grown on tissue culture-treated polycarbonate filters as previously described.

2.4. Degradation studies

Cell preparations containing type II, type I-like, or A549 cells in isotonic phosphate buffer were sonicated and the samples were adjusted to yield a final protein concentration of 1 mg ml^{-1} using BCA protein assay (Pierce, Rockford, IL). These samples were incubated with LHRH or [D-Ala^6]-LHRH at a final concentration of 0.85 μM for up to 3 h at 37°C in a shaker water bath. In some studies, enzyme inhibitors, captopril (30 μM), thiorphan (30 μM), and EDTA (30 μM), were also added to the incubation mixtures. The mixtures were sampled in aliquots of 100 μl at 0, 10, 30, 60, 90, 120, 150, and 180 min, and boiled for 5 min to stop the reaction. The resulting solutions were centrifuged at 13 000 rpm for 5 min to

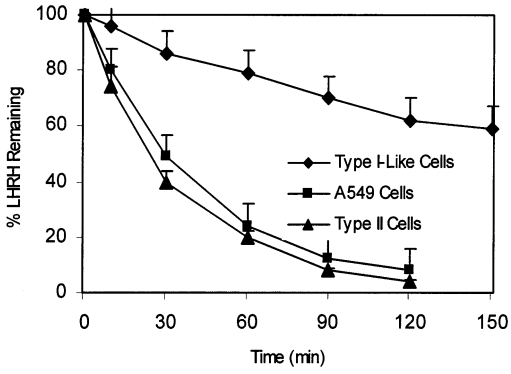


Fig. 1. Degradation profile of LHRH in type II, type I-like, and A549 cell preparations. Experiments were conducted in isotonic phosphate buffer at 37°C. The protein content in all cell preparations was adjusted to 1 mg ml^{-1} and the final concentration of LHRH was 0.85 μM. Separate studies showed that this concentration was in the linear range of the degradation kinetics of LHRH. Each data point represents the mean ± SE of four experiments.

precipitate any cellular debris, and 50 μl of the clear supernatant was collected and assayed by HPLC.

2.5. Peptide analysis

Analysis of LHRH, [D-Ala^6]-LHRH, and their degradation products were carried out using a reverse-phase HPLC method. The HPLC system consisted of a Waters 600E system controller (Waters Corp. Milford, MA, USA), Waters WISP 701B autosampler, Waters 486 tunable absorbance detector and Waters 746 data module. Sample separation was achieved using a Phenomenex bondclone C18 (150 × 3.9 mm) column and a linear gradient mobile phase consisting of 0.1% TFA and CH_3CN, (pH 3.0) as solvent A and 0.1% TFA as solvent B. The flow rate was 1 ml min^{-1} with a linear increase of solvent A from 10 to 30% within 40 min. LHRH and its fragments, LHRH 2–10, LHRH 3–10, LHRH 4–10, LHRH 6–10, and LHRH 7–10 were used as standards for the HPLC assay. The metabolites in the elutants were detected by UV at 215 nm. The quantitation of amounts of metabolites formed was determined using the method of least squares of the area under curve–concentration plot. A standard curve was generated for each experiment. The degradation of LHRH in various cell preparations was analyzed using first-order kinetics. Half-life for the degradation of the parent peptide was determined from the slope of the plot of log concentration of LHRH versus time.

3. Results

3.1. LHRH degradation in alveolar pneumocytes

The degradation profiles of LHRH in alveolar type II, type I-like, and A549 cell preparations are shown in Fig. 1. In all cell types, the degradation profiles appear to follow first order kinetics. The rate of LHRH degradation in type II cell preparations is comparable to that in A549 cells but is much slower in type I-like cells. Table 1 summarizes the degradation rate constants and half-lives of LHRH in these three cell preparations. No statistical difference was observed between the

Table 1
First-order rate constants and half-lives for the degradation of LHRH and [D-Ala^6]-LHRH in alveolar type II, type I-like, and A549 cell preparations*

	Type II cells[a]		Type I-like cells[b]		A549 cells	
	k (h^{-1})	$t_{0.5}$ (h)	k (h^{-1})	$t_{0.5}$ (h)	k (h^{-1})	$t_{0.5}$ (h)
LHRH	1.61 ± 0.13	0.43 ± 0.04	0.14 ± 0.02	4.99 ± 0.44	1.47 ± 0.08	0.47 ± 0.03
[D-Ala^6]-LHRH	0.22 ± 0.02	3.11 ± 0.25	0.05 ± 0.01	13.51 ± 1.03	0.20 ± 0.02	3.39 ± 0.41

* Each value indicates the mean ± SE of four different experiments.
[a] Freshly isolated type II cells.
[b] Type II cells at day 7 in culture.

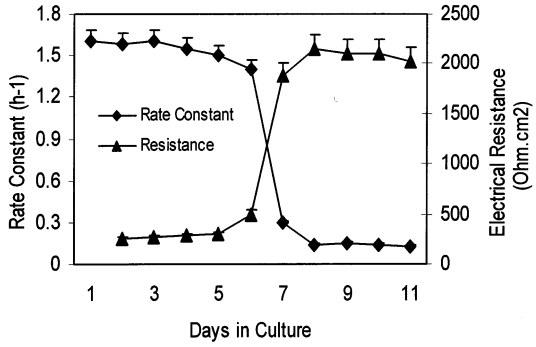


Fig. 2. Effect of cell differentiation on proteolytic activities of type II pneumocytes. Cells were cultured on microporous polycarbonate filters for up to 10 days. At indicated times, the cells were determined for their transepithelial electrical resistance and subsequently proteolytic activities using LHRH as a substrate. Each data point represents the mean ± SE of four experiments.

degradation rates of LHRH in type II cells and in A549 cells ($n = 4$, $P < 0.01$).

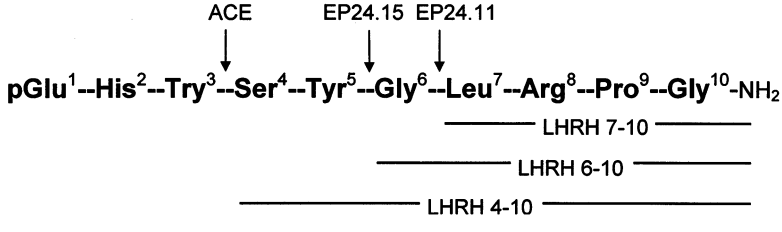
The degradation products of LHRH in the three cell preparations were identified by HPLC using known LHRH fragment standards. Three products were found in primary type II and A549 cell preparations, whereas only one product was detected in type I-like cell preparation. The products found in type II and A549 cell preparations were identified as LHRH 4–10, LHRH 6–10, and LHRH 7–10. In type I-like cell preparation, LHRH 7–10 was identified. Under the chromatographic conditions used in this study, the average retention times for LHRH and its metabolites are as follows: 8.7 min for LHRH 7–10, 16.5 min for LHRH 6–10, 21.8 min for LHRH 4–10, and 27.6 min for LHRH. The identity of LHRH metabolites was further confirmed using specific enzyme inhibitors in subsequent studies.

3.2. Effect of type II cell differentiation on proteolytic activities

The effect of type II cell differentiation on proteolytic activities was examined over a period of 10 days in culture. Transepithelial electrical resistance and cellular proteolytic activities for LHRH were determined each day during the culture period. Previous studies have shown that the transformation of type II cells into type I cells results in the formation of tight monolayers possessing high transepithelial electrical resistance (Cheek et al., 1989). Therefore, in this study, we used this parameter as an indicator for cellular differentiation and tight junction formation. Our results show that type II cells exhibited a sharp increase in electrical resistance on days 5–6 in culture (Fig. 2). The resistance values ranged from $\approx 250\ \Omega\ cm^2$ on day 2–5 up to $\approx 2100\ \Omega\ cm^2$ after day 6. This increase was accompanied by a sharp drop in cellular proteolytic activities, with the degradation rate constant of $\approx 1.4\ h^{-1}$ on day 5 and $\approx 0.3\ h^{-1}$ on day 6. These results suggest that the transformation of type II to type I cells results in a decrease in cellular proteolytic activities. A549 cells grown in culture showed no changes in both proteolytic activities ($k \approx 1.4\ h^{-1}$) and transepithelial electrical resistance ($\approx 260\ \Omega\ cm^2$) over a 10-day period. Morphologically, these cells maintained their cuboidal, type II-like appearance throughout the culture period (result not shown).

3.3. Effect of enzyme inhibitors on proteolytic activities

Various proteolytic enzymes have been shown to degrade LHRH in various tissues (Orlowski et



Scheme 1. Cleavage sites of LHRH by ACE and endopeptidases.

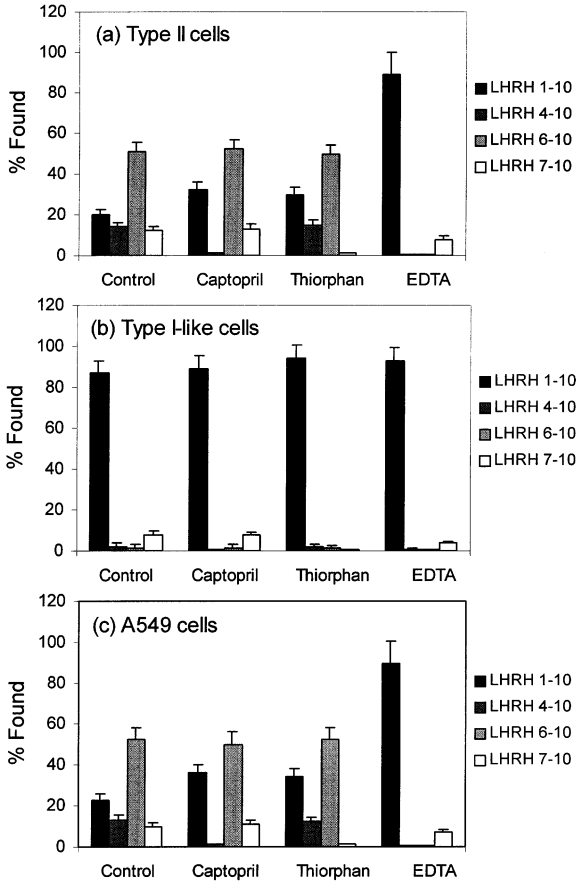


Fig. 3. Effect of enzyme inhibitors on the degradation of LHRH in type II, type I-like, and A549 cell preparations. Cell samples were incubated with LHRH in the presence of captopril (30 μM), thiorphan (30 μM), or EDTA (30 μM) for 60 min. LHRH 110 is the parent LHRH peptide. Each data point represents the mean ± SE of four experiments.

Table 2
Effect of enzyme inhibitors on the degradation half-lives (h) of LHRH in type II, type I-like, and A549 cell preparations[a]

	Type II cells	Type I-like cells	A549 cells
Control	0.43 ± 0.04	4.99 ± 0.44	0.47 ± 0.03
Captopril	0.61 ± 0.05	5.95 ± 0.46	0.68 ± 0.07
Thiorphan	0.57 ± 0.06	11.20 ± 1.09	0.64 ± 0.05
EDTA	5.95 ± 0.68	9.55 ± 0.07	6.58 ± 0.71

[a] Each value indicates the mean ± SE; $n = 4$.

al., 1983; Han et al., 1995). These enzymes and their cleavage sites are summarized in Scheme 1. To determine whether these and possibly other enzymes are responsible of LHRH degradation in alveolar pneumocytes, we used known enzyme inhibitors and studied their effect on LHRH degradation and metabolite formation. Fig. 3a and b show that captopril, a specific inhibitor of ACE (Turner et al., 1985), completely inhibited the formation of LHRH 4–10 fragment in type II and A549 cell preparations, thus indicating the presence of ACE and its activity in these two cell types. As indicated earlier, the LHRH 4–10 fragment was absent in the type I-like cell preparation (Fig. 3c). Fig. 3a–c also show that thiorphan, a specific inhibitor of EP24.11 (Turner et al., 1985), effectively inhibited the formation of LHRH 7–10 fragment in all three cell types, indicating the presence of EP24.11 in these cells. The role of EP24.15 in LHRH degradation was studied using EDTA, an EP24.15 inhibitor (Maggi et al., 1993). As shown in Fig. 3a–c, EDTA effectively inhibited the formation of LHRH 6–10 in all cell preparations, suggesting the role of EP24.15 in these cells. Furthermore, our results indicate that EDTA strongly inhibited ACE but had relatively little effect on EP24.11. Because of the possible non-specific effects of EDTA on endopeptidase activities, the role of EP24.15 in lung pneumocytes was further examined using an EP24.15-resistant peptide substrate, [D-Ala^6]-LHRH (see Section 3.4 below). The effects of EDTA and other inhibitors on the half-lives of LHRH in various pneumocytes are summarized in Table 2.

3.4. [D-Ala^6]-LHRH degradation in alveolar pneumocytes

[D-Ala^6]-LHRH, an active analog of LHRH in which L-Gly^6 is replaced by D-Ala, is resistant to degradation by EP24.15 (Flouret et al., 1987). In all pneumocytes tested, this peptide analog was found to be much more stable than the parent LHRH. The half-life of [D-Ala^6]-LHRH in these cells was 3–8 times greater than that of LHRH (Tables 2 and 3). In addition to resisting EP24.15 activity, this compound was also resistant to EP24.11 activity, as evidenced by the lack of

Table 3
Effect of enzyme inhibitors on the degradation half-lives (h) of [D-Ala^6]-LHRH in type II, type I-like, and A549 cell preparations*

	Type II cells	Type I-like cells	A549 cells
Control	3.11 ± 0.41	13.51 ± 1.44	3.40 ± 0.23
Captopril	13.51 ± 1.05	22.70 ± 2.06	16.98 ± 1.97
Thiorphan	3.72 ± 0.36	13.41 ± 1.19	3.97 ± 0.55
EDTA	22.75 ± 3.08	34.30 ± 3.07	22.03 ± 3.01

* Each value indicates the mean ± SE; $n = 4$.

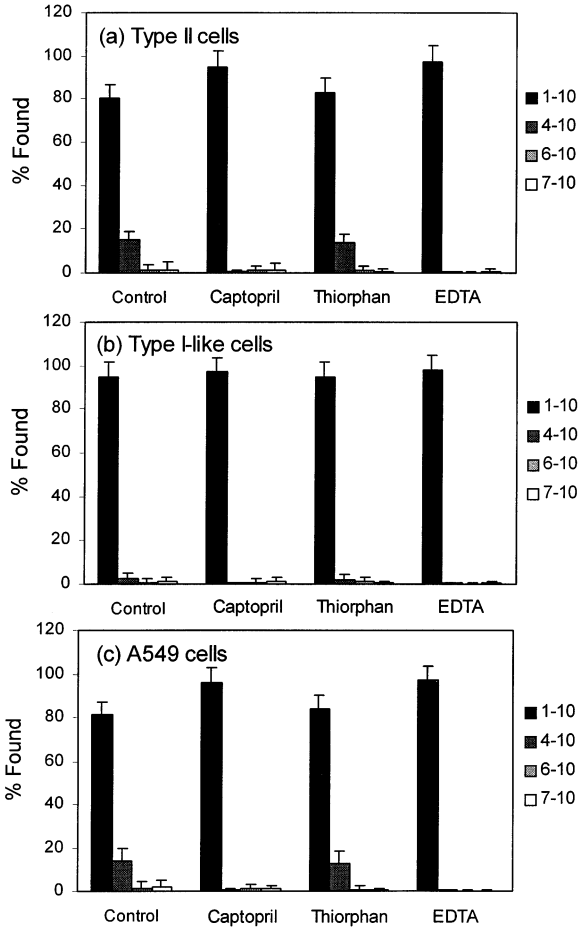


Fig. 4. Effect of enzyme inhibitors on the degradation of [D-Ala^6]-LHRH in type II, type I-like, and A549 cell preparations. The cell samples were incubated with [D-Ala^6]-LHRH in the presence of captopril (30 μM), thiorphan (30 μM), or EDTA (30 μM) for 60 min. Each data point represents the mean ± SE of four experiments.

fragments 6–10 and 7–10 in cell preparations (Fig. 4a–c). However, this compound was susceptible to ACE-mediated cleavage, which results in the formation of fragment 4–10 (Fig. 4a–c). Addition of ACE inhibitor (captopril or EDTA), but not EP24.11 inhibitor (thiorphan), to the cell preparations inhibited the formation of fragment 4–10, thus confirming that this fragment was indeed the degradation product of ACE.

4. Discussion

The present study demonstrates that the cells that constitute transport and enzyme barriers of the lung possess significant proteolytic activities. The degradation rate constants of LHRH in type II and type I-like cells were 1.61 ± 0.13 h^{-1} and 0.14 ± 0.01 h^{-1}, respectively. These values are, however, lower than those previously reported in other epithelial membranes including the rectal (8.01 ± 0.85 h^{-1}) and nasal (3.05 ± 0.02 h^{-1}) (Han et al., 1995). This finding is consistent with previous reports by our group (Wang et al., 1993) and others (Kashi and Lee, 1986) which demonstrated that the degradation rate of peptide enkephalin in alveolar epithelium (0.53 ± 0.04 h^{-1}) was lower than that in the nasal (2.55 ± 0.26 h^{-1}), rectal (3.68 ± 0.37 h^{-1}), buccal (3.45 ± 0.27 h^{-1}), vaginal (1.87 ± 0.23 h^{-1}), or ileal tissue (2.75 ± 0.19 h^{-1}). Similarly, in vivo studies by Patton and Platz (1992), Hoover et al. (1992), and Adjei and Garren (1990) also demonstrated that the pulmonary administration of human growth hormone, leuprolide, and several other peptides gave higher drug bioavailabilities than nasal or oral administration. Based on these studies, it appears that the lung exhibits lower proteolytic activities than most mucosal tissues and therefore represents an attractive alternative to peptide delivery.

Previous studies have shown that because of the presence of an N terminal pyroglutamate residue and a C terminal amide bond on LHRH, this compound is resistant to degradation by most exopeptidases such as aminopeptidases and carboxypeptidases (MacCann, 1977). The enzymatic degradation of LHRH is therefore governed by endopeptidases. The resistance to exopeptidases

and susceptibility to endonucleases of LHRH are also demonstrated in this study using alveolar pneumocytes. The major metabolites of LHRH in type II cells are LHRH 4–10, LHRH 6–10, and LHRH 7–10 fragments, whereas LHRH 7–10 fragment is the only metabolite in type I-like cells. The formation of LHRH 4–10 fragment suggests the role of ACE, which cleaves the Trp^3-Ser^4 bond. The formation of LHRH 6–10 and LHRH 7–10 fragments indicates that LHRH is also a substrate for EP24.15 and EP24.11. The EP24.15 cleaves LHRH at the central Tyr^5-Gly^6 bond, whereas the EP24.11 cleaves the Gly^6-Leu^7 bond. These results are in agreement with previous reports by Skidgel and Erdos (1984) and Molineaux et al. (1988). The presence of ACE and EP24.11 has also been reported in enterocytes (Bai and Amidon, 1992), while Han et al. (1995) found ACE, EP24.11, and EP24.15 in several other mucosal tissues. These studies indicate that these endopeptidases are widely distributed and play important roles in possessing biologically active peptides in various organs.

The observation of the lack of LHRH 4–10 and LHRH 6–10 fragments in type I-like cells suggests that the ACE and EP24.15 are either absent or inactive in this cell type. This finding has not been reported but is consistent with earlier findings (Erdos, 1984) demonstrating that the transformation of type II cells to type I cells is accompanied by a significant reduction in enzyme activities. This reduction in enzyme activities may be responsible for certain pathologic conditions specific to type I cells. For example, *Pneumocystis carinii* pneumonia (a characteristic infection of AIDS patients) results from the attachment of *P. carinii* to the type I cells (Yoneda and Walzer, 1980). This infection is type I cell-specific, as *P. carinii* rarely binds to type II cells. The greater susceptibility to injury associated with type I cells has also been attributed to the lack of enzymatic activities in this cell type (Simon and Kunkel, 1991).

Studies have shown that when type II cells are cultured, they lose those morphological and biochemical characteristics associated with type II phenotype. Expression of alkaline phosphatase, an enzyme marker of type II cells, decreases with time as type II cells are cultured (Edelson et al., 1988). Consistent with this finding, our results also show that type II cells exhibit a sharp decrease in proteolytic activities after 6 days in culture. We suggest that ACE and EP24.15 may further serve as differential markers for the differentiation of type II to type I cells.

5. Conclusion

This study demonstrates that alveolar epithelial cells, particularly the type II cells, exhibit significant enzyme activities against LHRH. However, their activities appear to be lower than those previously reported in most other epithelia. The transformation of alveolar type II to type I cells results in a 10-fold decrease in the overall proteolytic activities due to the loss of ACE and EP24.15 activities. The [D-Ala^6]-LHRH analog is 3–8 times more enzymatically stable than LHRH because of its resistance to EP24.15 and EP24.11 degradation. The A549 cells exhibited similar enzyme activities as the type II cells, both in terms of the rate and pattern of degradation. This continuous lung cell line may be useful as a lung type II cell model for drug metabolism studies. However, this cell line does not form tight monolayers and therefore may not be suitable for drug absorption studies. Further development of continuous cell lines with type I characteristics should contribute to membrane transport studies of pulmonary administered drugs.

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