Enzymatic Degradation of Luteinizing Hormone Releasing Hormone (LHRH)/[D-Ala^6]-LHRH in Lung Pneumocytes

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Purpose. To investigate the cellular proteolytic activities of various lung pneumocytes using luteinizing hormone releasing hormone (LHRH) and [D-Ala^6]-LHRH as model peptide substrates.

Methods. HPLC analysis was used to investigate the degradation kinetics of LHRH/[D-Ala^6]-LHRH and to identify their degradation products in isolated lung pneumocytes.

Results. Pulmonary macrophages exhibited the strongest proteolytic activity against LHRH)/[D-Ala^6]-LHRH, followed by type II and type I-like pneumocytes. Three major degradation products of LHRH, namely LHRH 4–10, LHRH 6–10, and LHRH 7–10, were identified in macrophages and type II pneumocytes, whereas in type I-like pneumocytes only the LHRH 7–10 was found. Co-incubation of the cells with known enzyme inhibitors including captopril (an ACE inhibitor), thiorphan (an EP24.11 inhibitor), and EDTA (an EP24.15 inhibitor) inhibited the formation of LHRH 4–10, LHRH 7–10, and LHRH 6–10 respectively. 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.

Conclusions. ACE, EP24.11, and EP24.15 are the major enzymes responsible for the degradation of LHRH in macrophages and type II pneumocytes. The magnitude of peptidase activities in these cell types are: EP24.15 > EP24.11 ≈ ACE. No EP24.15 or ACE activity was observed in type I-like pneumocytes and only a weak EP24.11 activity was detected.

KEY WORDS: peptides; lung; degradation; LHRH; epithelial cells; macrophages.

INTRODUCTION

The pulmonary route of administration represents an attractive alternative to the oral route for systemic delivery of peptide and protein drugs. Peptides and proteins that have been investigated for systemic delivery via the pulmonary route include human growth hormone, insulin, α1-antitrypsin, leuprolide, and vasopressin (see Ref. 1 for review). These peptides apparently cross the air-blood barrier in sufficient quantities to elicit systemic biological effects when aerosolly delivered to the lung. While the pulmonary route of administration can deliver the biologically active form of peptide into circulation, this method of administration is still considerably less efficient than the injectable method. One key barrier limiting its efficiency is the presence of proteolytic activities in numerous lung pneumocytes. To date, most enzymatic studies in the lung have been conducted using intact or perfused mammalian lungs. While this method has provided useful information concerning the overall enzymatic activity of the lung, it does not provide specific information concerning the precise location and relative contribution of various lung cell types in the degradation process. In this study, we utilize various lung cell systems and evaluate their specific contribution in the overall degradation process. We also examine the nature and extent of proteolytic enzymes in these lung cells using LHRH and [D-Ala^6]LHRH as model peptides.

In the lung, the pulmonary alveolar region represents the major absorptive site for drugs. The average human alveolus has a surface area of ≈207,000 μm^2 and is covered on average by 40 epithelial type I cells, 67 epithelial type II cells, and 12 alveolar macrophages which attach to the top of the epithelium (2). These three cell types are principally responsible for the enzymatic activity presented in the pulmonary airspace. The macrophages are professional phagocytes whose primary function is to digest and detoxify foreign substances in the pulmonary airspace (3). Their role in the defense against pulmonarily-administered peptide drugs has been predicted, however this role has not been investigated. The epithelial type I and II cells cover the alveolar lining of the lung and present the permeability barrier to inhaled substances (4). To enter the systemic circulation, peptide drugs must cross this barrier while being subjected to enzymatic degradation. The nature and extent of proteolytic enzymes in these cells as well as in macrophages have not been systemically investigated. The present study was undertaken to address the following questions: 1) what cell type is primarily responsible for the degradation of peptides in the airspace; 2) what is the relative contribution of each specific cell type in the overall degradation process; and 3) what are the major enzymes responsible for the degradation of peptides in lung pneumocytes. To answer these questions, we incubated LHRH/[D-Ala^6]-LHRH with various lung cells from rats. Lung macrophages were obtained by bronchoalveolar lavage and type II pneumocytes were prepared by enzymatic digestion. Because of the lack of appropriate methods to isolate and purify type I pneumocytes, the present study utilized type II pneumocytes and cultured them on tissue culture-treated solid supports. These cultured cells formed tight monolayers and exhibited morphologic characteristics of type I cells.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats weighing between 100–150 g (Hilltop Labs. Scottsdale, PA) were used. LHRH, [D-Ala^6]-LHRH, LHRH 7–10 fragment, LHRH 4–10 fragment, LHRH 3–10 fragment, LHRH 2–10 fragment, captopril, thiorphan, and disodium ethylene diaminetetracetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO). LHRH 6–10 fragment was custom synthesized by Quality Control Biochemical Inc. (Hopkinton, MA).

Preparation of Pneumocytes

Alveolar macrophages were harvested from rats by bronchoalveolar lavage. The animals were anesthetized by intraperi-

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toneal injection of sodium pentobarbital (0.2 g/kg). The trachea was cannulated and the lungs were lavaged with phosphate-buffered saline (PBS). Lavage cell suspensions were centrifuged and the cell pellets were then washed by alternate resuspension and centrifugation in PBS. Cell viability was measured by trypan blue exclusion and was typically found to be >95%. Type II pneumocytes were prepared according to the method previously described (5). After bronchoalveolar lavage, the lungs were excised and filled with PBS containing elastase (40 units/ml, type I; U.S. Biochmical) and DNase (0.006%; Sigma) and incubated at 37°C for 20 min. 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, and the second cell band from the surface was collected and resuspended in 1:1 F12 and Eagle's modified essential medium. The cell suspension yielded 5×10^6 cells/rat with viability >95%. 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 of Cheek *et al.* (6) with modifications. Briefly, freshly isolated type II cells were plated onto 0.4-μm-pore, 0.3-cm^2 tissue-treated polycarbonate filters (Costar, Cambridge, MA) at 1×10^6 cells/cm^2 on 24-well plates. The cells were maintained at 37°C in 5% CO_2 and the nutrient medium (see above) was changed every 48 h. Cell confluency was monitored by electrical resistance measurements using the Millicell ERS device (Millipore, Bedford, MA) and scanning electron microscopy (5,6). 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 monolayers exhibited an increase in electrical resistance from 246 ± 43 Ω · cm^2 on day 1 up to 2,100 ± 225 Ω · cm^2 on day 6. These morphologic and functional changes are consistent with those observed in developing type I cells *in vivo* (6). Using monoclonal antibodies specific to type I pneumocytes, Danto *et al.* (7) previously demonstrated that type II cells grown on filter developed a phenotypic characteristic of type I cells. In this study, type II cells grown on filters for 6 days were used to represent type I pneumocytes.

Degradation Studies

Pneumocyte samples containing macrophages, type II cells, or type I-like cells in isotonic phosphate buffer were sonicated and the sample solutions were adjusted to yield a final protein concentration of 1 mg/ml. In studies designed to investigate the cellular distribution of LHRH degrading enzymes, intact cells, their membrane fractions, and trypsinized cells were sometimes used. The membrane fractions were prepared by differential centrifugation as previously described (8). Trypsinization was performed by treating the cells with 0.25% trypsin in PBS for 10 min at 37°C. All test samples were incubated with LHRH or [D-Ala^6]-LHRH at a final concentration of 0.846 μM for up to 3 hours at 37°C. The incubation mixtures were sampled in aliquots of 100 μl at indicated times and boiled for 5 min to stop the reaction. The resulting solutions were centrifuged at 13,000 rpm for 5 min to precipitate any cellular debris, and 50 μl of the clear supernatant was collected and assayed by HPLC. In some studies, enzyme inhibitors, captopril (30 μM), thiorphan (30 μM), and EDTA (30 μM), were also added to the incubation mixtures.

HPLC Analysis

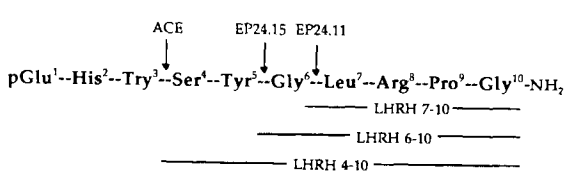
The separation and identification of LHRH/[D-Ala^6]-LHRH and their metabolites were achieved using a reverse-phase HPLC method. Sample separation was achieved using a Phenomenex bondclone C_{18} (150 × 3.9 mm) column and a linear gradient mobile phase consisting of 0.1% TFA and CH_3CN (pH 3.0) as solvent A and 0.1% TFA as solvent B. The flow rate was 1 ml/min with a linear increase of solvent A from 10% to 30% within 40 min. LHRH and its fragments (see above) were used as standards for the HPLC assay. The metabolites in the elutants were detected by UV at 215 nm.

RESULTS AND DISCUSSION

Chromatographic Determination of LHRH Degradation

HPLC analysis was conducted to identify the degradation products of LHRH in pneumocyte preparations. Three major degradation products were found in macrophage and type II cell preparations, whereas only one product was detected in type I-like cell preparation. Using known LHRH fragment standards, the degradation products in the macrophage and type II cell preparations were identified as LHRH 4–10, LHRH 6–10, and LHRH 7–10. In type I-like cell preparation, the degradation product was identified as LHRH 7–10. Under the chromatographic conditions given under the Materials and Methods, 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 kinetics of metabolite formation and their identity were further studied using specific enzyme inhibitors in subsequent studies.

Previous sudies have shown that due to 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 (9). The enzymatic degradation of LHRH is therefore governed by endopeptidases. The major endopeptidases involved in the degradation of LHRH have been characterized in the pituitary and brain (10) and in other mucosal tissues (11). Scheme 1 summarizes the major enzymes involved in the proteolytic cleavage of LHRH in these tissues. In the present study, we show that the degradation products of LHRH are similar in macrophages and type II cells. The formation of LHRH 4–10 fragment in these cells suggests the role of angiotensin converting enzyme (ACE), which cleaves the Trp^3-Ser^4 bond, in LHRH degradation. The formation of LHRH 6–10 and LHRH 7–10 fragments indicates that LHRH is also a substrate for endopeptidase EP24.15 and EP24.11. The EP24.15



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

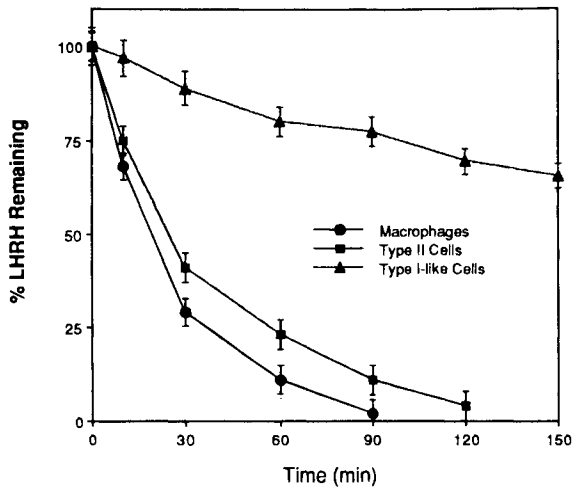


Fig. 1. Degradation profile of LHRH in various pneumocyte preparations. Incubation was conducted in isotonic phosphate buffer at 37°C using sonicated cell preparations. The protein concentration in all cell preparations was adjusted to 1 mg/ml. Each data point represents the mean ± S.E. of four experiments.

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 (12) and Molineaux *et al.* (13). Interestingly, the degradation of LHRH in type I-like cell preparation does not involve the formation of LHRH 4–10 and LHRH 6–10 fragments. These results suggest that ACE and EP24.15 are either absent or inactive in this cell type. This finding has not been previously reported but is consistent with earlier findings (14) which demonstrate that the transformation of type II to type I cells is accompanied by a significant reduction in enzyme activities. Similarly, Edelson *et al.* (15) showed that the activity of alkaline phosphatase, an enzyme marker of type II cells, decreased with time as type II cells were cultured *in vitro*.

LHRH Degradation in Alveolar Pneumocytes

The degradation profiles of LHRH in alveolar macrophage, type II and type I-like cell preparations are shown in Fig. 1. In all cell types, the degradation profiles appear to follow first order kinetics. Table I summarizes the rate constants of LHRH in the three cell preparations. The rank order of proteolytic activity in these cells are: macrophages > type II cells > type I-like cells. The rate of degradation of LHRH in intact

Table I. Degradation Rate Constant (h^{-1}) of LHRH After Incubation with Various Pneumocyte Preparations[a]

	Macrophages	Type II Cells	Type I-Like Cells
Sonicated Cells	0.35 ± 0.02	0.28 ± 0.01	0.03 ± 0.01
Intact Cells	0.37 ± 0.02	0.25 ± 0.02	0.03 ± 0.01
Membrane Fraction	0.38 ± 0.02	0.24 ± 0.01	0.03 ± 0.01
Trypsinized Cells	<0.02 ± 0.01[b]	<0.02 ± 0.01[b]	<0.01 ± 0.01[b]

[a] Each value represents mean ± S.E. of four different experiments.
[b] Indicates significant difference over sonicated controls, $p < 0.01$.

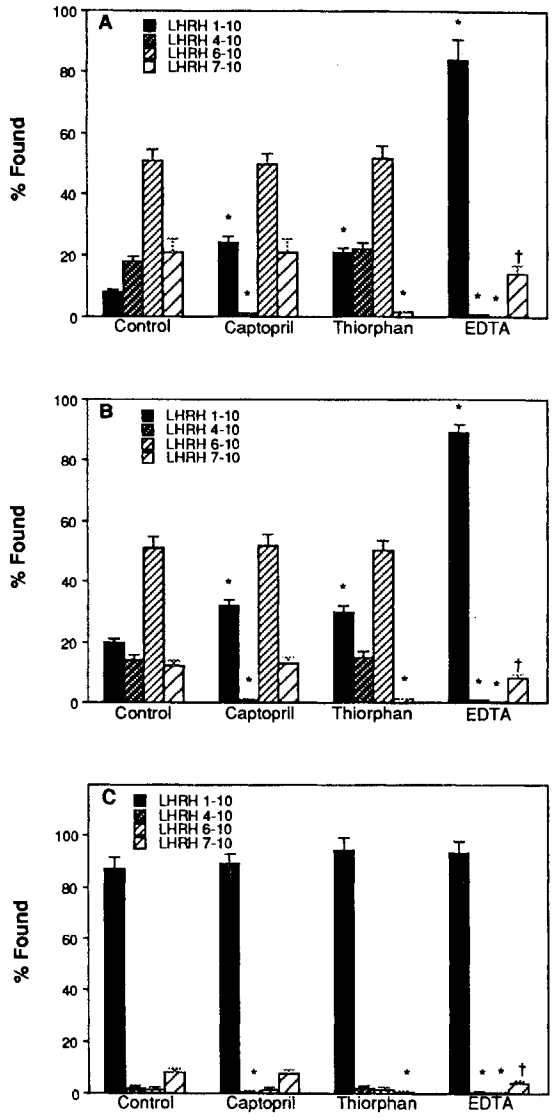


Fig. 2. Inhibitory effects of various enzyme inhibitors on LHRH degradation in alveolar macrophage (A), type II pneumocyte (B), and type I-like pneumocyte preparations (C). The cell samples were incubated with LHRH in the presence of captopril (30 μM), thiorphan (30 μM), or EDTA (30 μM) for 60 min. LHRH 1–10 is the parent LHRH peptide. Each data point represents the mean ± S.E. of four experiments. *$p < 0.01$, †$p < 0.05$ compared with control.

pneumocytes or in membranous fractions is not significantly different from that in sonicated cells (Table I), suggesting that the enzymes responsible for LHRH degradation are likely to be membrane-bound. In addition, pretreatment of cells with trypsin to remove membrane-bound enzymes resulted in a dramatic decrease in LHRH degrading enzyme activities.

In a previous report which compared the enzymatic stability of LHRH in nasal and other mucosal tissue homogenates (11), it was found that the half life of LHRH in the nasal tissue was about 19 min, and that in the rectal and vaginal tissues was about 7 min and 90 min, respectively. The value in the nasal tissue is comparable to that observed in lung macrophages (18 min), but is lower than that in type II (24 min) and type I-like pneumocytes (249 min) (data extracted from Table I).

Table II. Effect of Enzyme Inhibitors on the Degradation Half Lives (min) of LHRH in Various Pneumocyte Preparations[a]

	Macrophages	Type II Cells	Type I-Like Cells
Control	18.1 ± 0.7	24.4 ± 0.9	249.7 ± 8.3
Captopril	29.1 ± 1.3[b]	36.5 ± 1.2[b]	346.8 ± 12.8[b]
Thiorphan	26.6 ± 0.7[b]	34.5 ± 1.1[b]	672.0 ± 23.3[b]
EDTA	238.4 ± 6.8[b]	356.8 ± 13.1[b]	572.9 ± 16.8[b]

[a] Each value represents mean ± S.E. of four different experiments.
[b] Indicates significant difference over controls, $p < 0.01$

Because over 95% of the total pulmonary surface area are covered with type I pneumocytes, these data suggest that the degradation rate of LHRH in the distal lung may be lower than that in the nasal tissue. Indeed, our studies using whole lung tissue homogenates demonstrate that the half life of LHRH in this tissue is about 31 min (unpublished data). This observation is supported by earlier findings by our group (5) and others (16) which demonstrated that the degradation rate of peptide enkephalin was lower in the lung tissue than in the nasal, rectal, or intestinal tissue. Likewise, studies by Patton *et al.* (1) and Adjei and Garren (17) also demonstrated that the pulmonary administration of human growth hormone and leuprolide 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 site for peptide delivery.

The responsible enzymes for the degradation of LHRH were further characterized using known enzyme inhibitors to probe the specific enzyme activities (Fig. 2). Captopril, a specific inhibitor of ACE (18), completely inhibited the formation of LHRH 4–10 fragment in macrophage and type II cell preparations (Figs. 2A and 2B), thus confirming 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 1 h after incubation (Fig. 2C). Figs. 2A–2C also show that thiorphan, a specific inhibitor of EP24.11 (16), effectively inhibited the formation of LHRH 7–10 in macrophages, type II and type I-like cells, thus confirming the presence of EP 24.11 in these cells. The role of EP24.15 in LHRH degradation was studied using EDTA (an EP24.15 inhibitor) (19), and an EP24.15-resistant substrate, [D-Ala^6]-LHRH. As shown in Figs. 2A–2C, EDTA effectively inhibited the formation of LHRH 6–10 in all pneumocyte preparations, suggesting the role of EP24.15 in these cells. Furthermore, our results also indicated that EDTA strongly inhibited ACE but had relatively little effect on EP24.11. These results are in good agreement

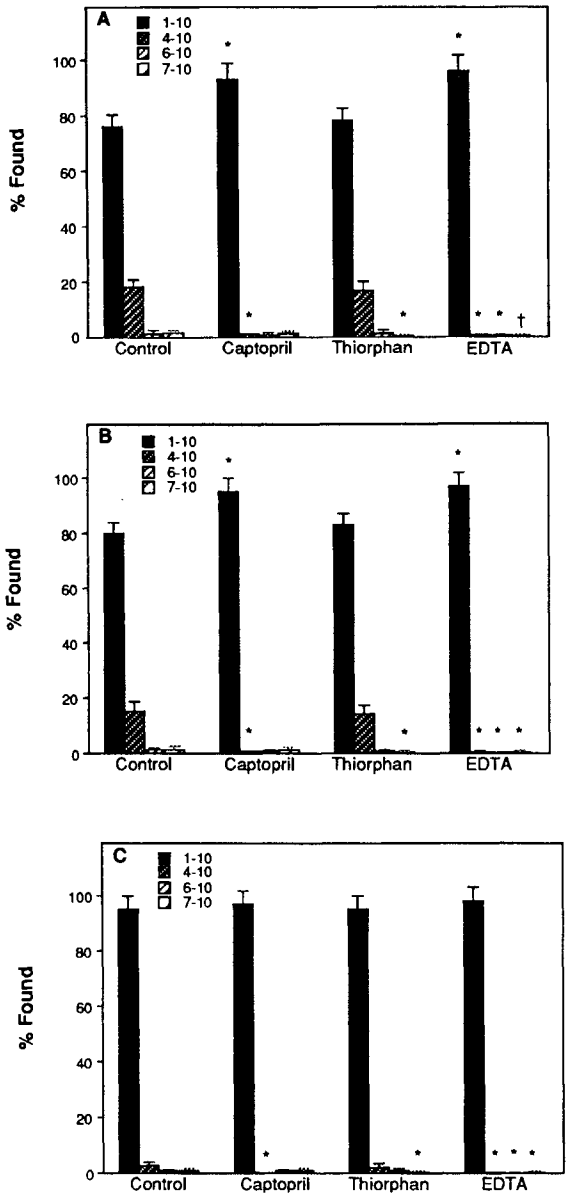


Fig. 3. Inhibitory effects of various enzyme inhibitors on the degradation of [D-Ala^6]-LHRH in alveolar macrophage (A), type II pneumocyte (B), and type I-like pneumocyte preparations (C). The cell samples were incubated with LHRH in the presence of captopril (30 μM), thiorphan (30 μM), or EDTA (30 μM) for 60 min. Each data point represents the mean ± S.E. of four experiments. *$p < 0.01$, †$p < 0.05$ compared with control.

Table III. Effect of Enzyme Inhibitors on the Degradation Half Lives (min) of [D-Ala^6]-LHRH in Various Pneumocyte Preparations[a]

	Macrophages	Type II Cells	Type I-Like Cells
Control	151.1 ± 6.7	24.4 ± 0.9	809.7 ± 28.3
Captopril	572.1 ± 19.3[b]	810.5 ± 24.2[b]	1365.8 ± 38.8[b]
Thiorphan	167.6 ± 5.7	223.5 ± 7.1[b]	772.0 ± 21.3
EDTA	1018.4 ± 36.8[b]	1356.8 ± 43.1[b]	2052.9 ± 56.8[b]

[a] Each value represents mean ± S.E. of four different experiments.
[b] Indicates significant difference over controls, $p < 0.01$.

with previous reports by Maggi *et al.* (19) and Han *et al.* (11). Because of the non-specific effects of EDTA on endopeptidase activities, the role of EP24.15 in lung pneumocytes was further examined using [D-Ala^6]-LHRH (see results below). The effects of EDTA and other inhibitors on the half lives of LHRH in various pneumocytes are summarized in Table II.

[D-Ala^6]-LHRH Degradation in Alveolar Pneumocytes

[D-Ala^6]-LHRH, a superactive analog of LHRH in which L-Gly^6 is replaced by D-Ala, is resistant to degradation by EP24.15 (20). *In vivo* studies by Nakamura *et al.* (21) showed

that this compound was 4.8 times more biologically active than LHRH. The results of this study also demonstrated that the half lives of [D-Ala^6]-LHRH in various pneumocytes were 3–8 times greater than LHRH (Tables II, III). In addition to resisting EP24.15 activity, this compound was also resistant to EP24.11 activity, as indicated by the absence of fragments 6–10 and 7–10 in the cell preparations (Figure 3A–3C). However, the compound was susceptible to ACE-mediated cleavage, leading to the formation of fragment 4–10 (Figure 3A–3C). An addition of ACE inhibitor (captopril or EDTA), but not EP24.11 inhibitor (thiorphan), to the cell preparations inhibited the formation of fragment 4–10, confirming that this fragment was indeed the metabolite of ACE.

CONCLUSIONS

This study demonstrates that lung pneumocytes are capable of degrading LHRH/[D-Ala^6]-LHRH in significant quantities. The rank order of proteolytic activities in these cells are: alveolar macrophages > type II cells > type I-like cells. The major enzymes responsible for the degradation of LHRH are EP24.15, EP24.11, and ACE, with the first enzyme being the most active in macrophages and type II pneumocytes. The EP24.15 and EP24.11 are inactive in type I-like pneumocytes, suggesting that the transformation of type II cells to type I cells results in decreased enzyme activities. Because macrophages and type II pneomocytes represent the major cells for the degradation of LHRH and because these cells exhibit strong EP24.15 activity, therapeutic strategies aimed at improving the biologic stability of peptides should therefore be focused on overcoming the EP24.15 activity. In this regard, [D-Ala^6]-LHRH presents a clear advantage over LHRH due to its resistance to EP24.15 degradation. Furthermore, this peptide is also resistant to EP24.11. The use of enzyme inhibitors to improve the stability of peptides, while offering a potentially effective alternative to peptide analogs, may suffer from adverse side effects, and thus their use must be carefully evaluated.

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