Pathways and Kinetics of Deslorelin Degradation in an Airway Epithelial Cell Line (Calu-1)

Kavitha Koushik,[1] Gangadhar Sunkara,[2] Peter Gwilt,[1] and Uday B. Kompella [1,3]

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Purpose. The objective of this study is to investigate the pathways and kinetics of degradation of deslorelin, pGlu[1]-His[2]-Trp[3]-Ser[4]-Tyr[5]-D-Trp[6]-Leu[7]-Arg[8]-ProNHEt[9] (Des1-9), in a human airway epithelial cell line (Calu-1).

Methods. The degradation of deslorelin in membrane and cytosolic fractions of Calu-1 cells was studied at 37°C up to 24 h. The degradation products were separated using HPLC and identified by amino acid analysis, sequencing, and mass spectrometry. The rate constants for deslorelin degradation and the formation of degradation products were determined by fitting the concentration vs. time data to pharmacokinetic models using WinNonlin™. The effect of enzyme inhibitors, captopril, phosphoramidon, and disodium EDTA on deslorelin degradation was also assessed.

Results. Des1-3, Des4-9, and Des5-9 were the deslorelin fragments detected in the membrane fraction. Apart from these degradation products, Des5-7 was also detected in the cytosolic fraction. The deslorelin degradation was 8.5 times faster in the cytosolic fraction compared to the membrane fraction. The disappearance of deslorelin and the kinetics of degradation products could be explained by simple 2 compartment iv bolus model and 1 compartment absorption model, respectively. EDTA and captopril decreased deslorelin degradation in the membrane and cytosolic fractions.

Conclusions. Deslorelin is initially cleaved at the 3-4 bond in the membrane and cytosolic fractions, possibly by a metalloendopeptidase and/or angiotensin converting enzyme, with the degradation being more rapid in the cytosol.

KEY WORDS: deslorelin; Calu-1 cells; degradation pathways; membrane, cytosol.

INTRODUCTION

Deslorelin, a nonapeptide with the amino acid sequence, pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-ProNHEt, is a leutinizing hormone releasing hormone (LHRH) agonist that is 144 times more potent than the native LHRH (1). Deslorelin is of therapeutic value in the treatment of endometriosis and uterine fibroids. In addition, it is useful in the treatment of precocious puberty and breast and prostate cancers (2). Respiratory delivery either by the nasal route or by inhalation through the deep lungs is an attractive therapeutic option for the non-invasive delivery of peptide drugs. Currently several peptide drugs including salmon calcitonin, buserelin, nafarelin, and desmopressin are available as nasal sprays. However, due to presystemic elimination by enzymatic degradation and poor membrane permeability, the nasal bioavailability of these peptides is less than 10% (3,4). Therefore, to increase the bioavailability, the pulmonary route is being investigated for the delivery of peptide drugs (5). The enzymatic degradation of the peptides in the airway epithelium, however, is a limiting factor for the bioavailability of peptides administered by way of this route. Thus, peptides administered by the nasal and the respiratory route would be degraded by the epithelial peptidases.

To overcome the enzymatic barriers for the respiratory delivery of peptide drugs, several enzyme inhibitors such as bestatin, amastatin, and α-aminoboronic acid have been used (3,4). In addition, absorption enhancers, such as fusidic acids, bile salts, and fatty acids, which have enzyme inhibitory action, have been used (3,4). All of these agents however, have a direct impact on the nasal membrane and result in toxicity upon chronic therapy. Another strategy to decrease the enzymatic degradation of peptide drugs has been to use cyclodextrins, which form a complex with the peptide and prevent the degradation. Our previous studies indicated that hydroxypropyl β-cyclodextrin (HPβCD) binds to deslorelin with a low affinity (Ka = 125 M^{-1}) and this could be one of the reasons for the moderate protection offered by HPβCD toward deslorelin degradation by α-chymotrypsin (6). Therefore, there is a need to explore new strategies to improve the delivery of deslorelin by way of the respiratory route. This study aims at fulfilling this need by understanding the degradation pathways of deslorelin.

Because degradation products within a system exist in a dynamic equilibrium of formation and degradation, a dissection of the formation and elimination kinetics will aid in understanding the sequential degradation, the stability of primary degradation products, and the abundance of particular peptidases within that system (7). In addition, a degradation product with higher affinity for the degrading enzyme could act as a competing substrate, undergo preferential degradation, and protect the parent drug (8). Therefore, a clear identification of the degradation products and a study of their kinetics in addition to drug degradation kinetics will help identify such potentially useful products. Using excised tissues, the authors observed that deslorelin is degraded to an extent of 50% or less in the nasal, corneal, and conjunctival tissues (9,10,11). Based on these studies, wherein the peptide primarily encountered the membrane region, it was speculated that the membrane bound enzymes could degrade deslorelin. In addition, ouabain and 2,4 dinitrophenol, which impair active cellular uptake of solutes, slightly decreased deslorelin degradation, suggesting the involvement of intracellular peptidases in deslorelin degradation. The rate and extent of deslorelin degradation in membrane and cytosolic fractions, however, remains to be investigated.

To investigate deslorelin degradation in the membrane and cytosolic fractions of the respiratory epithelium, we used Calu-1, a human airway epithelial cell line derived from human non-small-cell lung carcinomas. These cells secrete mucus, possess cilia, and have extensive endopeptidase activity, similar to the normal airway epithelium present in the nose and the bronchial tract (12). In addition, Calu-1 cells express neutral endopeptidase (NEP; also known as EC 3.4.24.11), which is a widely distributed membrane bound enzyme that

[1] Department of Pharmaceutical Sciences, 986025 University of Nebraska Medical Center, Omaha, Nebraska 68198.
[2] Department of Clinical Pharmacokinetics, Novartis Pharmaceuticals Corporation, East Hanover, New Jersey 07936.
[3] To whom correspondence should be addressed. (e-mail: ukompell@unmc.edu)

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hydrolyzes many biologically important endogenous peptides (12).

The objectives of this study were: to identify the major degradation products of deslorelin, to determine the kinetics of deslorelin degradation and the formation of major deslorelin degradation products, and to determine the influence of peptidase inhibitors on deslorelin degradation in the membrane and cytosolic fractions of Calu-1 cells.

MATERIALS AND METHODS

Chemicals

Cell culture materials and reagents were obtained from Gibco (Grand Island, NY, USA) and Becton Dickinson Labware (Franklin Lakes, NJ, USA). Deslorelin was a gift from Balance Pharmaceuticals, Inc. (Santa Monica, CA, USA). Calu-1 cells were obtained from ATCC (Manassas, VA, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). On identification, the deslorelin degradation fragments, pGlu-His-Trp, Ser-Tyr-D-Trp-Leu-Arg-ProNHEt and -Tyr-D-Trp-Leu-Arg-ProNHEt were synthesized and purified at a peptide synthesis facility at the University of Nebraska Medical Center (Omaha, NE, USA).

Cell Culture

Calu-1 cells were grown in culture as previously described (13). Briefly, mycoplasma free Calu-1 cells (ATCC number: HTB-54) were grown in McCoy's 5A Medium with 10% fetal bovine serum supplemented with 1% sodium pyruvate, L-glutamine and 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate. The cells were incubated in a 5% CO_2 and 95% O_2 atmosphere at 37°C and subcultured upon attaining confluence on days 6-8. All experiments were performed using day 8 cells of passages 28–35, grown in 75 or 175 cm^2 culture flasks.

Isolation of Membrane and Cytosolic Fractions of Calu-1 Cells

The membrane and cytosolic fractions of Calu-1 cells were separated using a previously described method (14). Briefly, cells were detached from culture flasks by trypsinization and disrupted in an ice-cold assay buffer (pH 7.4) using a sonicating cell disruptor (Branson Sonifier®, Cell Disruptor 200, Branson Ultrasonics Corporation, Danbury, CT, USA). After centrifugation at 1000 *g* for 10 min on a Sorvall RTH250 (Dupont, Sorvall Products®, Newtown, CT, USA), the supernatant was ultracentrifuged at 100,000 *g* (Beckman LE 80k, Beckman Instruments, Palo Alto, CA, USA) for 60 min. The resulting supernatant was the cytosolic fraction and it was further purified by ultracentrifugation at 100,000 *g*. The pellet was re-dispersed in ice-cold buffer, re-centrifuged at 100,000 *g* for 60 min, and the supernatant was discarded to remove any contaminating soluble fractions. All steps were carried out at 4°C and the pellet obtained was used as the membrane fraction. The protein concentrations of the membrane and the cytosolic fractions were determined using a Pierce BCA™ protein assay kit (Pierce Products, Rockford, IL, USA). The protein concentration in all the studies was adjusted to 1 mg/ml using assay buffer.

Deslorelin Degradation in Cell Fractions

The membrane and cytosolic fractions of Calu-1 cells were isolated as described above and used immediately for the degradation studies. Deslorelin (1 mg/ml) was incubated with these fractions at a protein concentration of 1 mg/ml in assay buffer at 37°C for 24 h. Aliquots of the reaction mixture were removed at various intervals and the reaction was stopped by adding an equal volume of a mixture of acetonitrile and water (50:50 v/v). The samples were frozen until analysis. Degradation studies were also conducted in the presence or absence of 1% disodium EDTA, 100 μm captopril, and 2 μm phosphoramidon.

Isolation of Deslorelin Degradation Products

The degradation products were isolated by high-pressure liquid chromatography using a Waters HPLC system comprising of a Waters 600 S controller, Waters TM 616 solvent delivery pump, and a Waters 717 plus auto injector. The peptide and fragments were detected using a Waters 996 PDA detector set at a wavelength of 220 nm and the peak areas were integrated using Millennium software (version 2.15.01). A Vydac® (VYDAC, Hesperia, CA) protein-peptide C-18 column, (218TP54; 250 × 4.6 mm), with a particle diameter of 5 μm and a pore size of 300 Å was used. Gradient elution was run with A) 0.1% TFA in Acetonitrile and B) 0.1% TFA in distilled water, at a flow rate of 1 ml/min. Elution started with an isocratic flow of 100% B for 10 min followed by a convex increase of A from 0–30% over 40 min and holding it at 30% for 10 min. The column was then re-equilibrated at 100% B for 10 min before the next injection. The fractions of the degradation fragments thus separated were collected for further purification. Collections of fragments were timed to coincide with appearance of peak in the detector. To ensure high concentrations for analysis and low contamination, collections were started at half peak height and 200 μl was collected for every degradation product.

Identification of Degradation Products

The degradation product fractions separated by HPLC as described above, were further purified using a HPLC. For the purification, the Waters HPLC system as described above was used. However, the elution was an isocratic method with the mobile phase consisting of 30% acetonitrile and 70% of 0.1% trifluoroacetic acid in distilled water delivered at the rate of 1 ml/min. A microsorb C-18 column (250 × 4 mm) with a particle diameter of 5 μm and a pore size of 100 Å from Rainin Instruments (Emeryville, CA, USA) was used. The fragments were collected as described in the previous section and were identified by amino acid analysis (Beckman 6300 Amino Acid Analyzer), peptide sequencing, and electrospray mass spectrometry employing fast atom bombardment (Nebraska Center for Mass Spectrometry, Lincoln, NE, USA). The deslorelin degradation products were quantified using pure synthesized degradation fragments as standards.

Data Analysis

To characterize the degradation of deslorelin and the subsequent formation of degradation products, the concentration vs. time profiles of deslorelin and its degradation

products were fitted to pharmacokinetic models using WinNonlin™ (Version 1.5, Scientific Consulting, Inc.). A two compartment model with bolus input and elimination from central compartment, intercompartmental microconstants, no lag time, and first order elimination (Fig. 1a) was used to characterize the concentration-time profiles of deslorelin in the membrane and cytosolic fractions. The degradation product formations were analyzed using a simple one-compartment oral absorption model with first order rate constants and no lag time, as illustrated in Fig. 1b. Using this model and uniform weighting for all data points, the mean data for the formation of primary and secondary degradation products were fitted by assuming the total amount of deslorelin added as the dose available for degradation. The compartmental model parameters, the incubation volume (V), the amount of deslorelin (D), and intercompartmental rate constants (K10, K01, K12 and K21) were used to calculate the parameters in the following equations:

$$C(t) = A * (e^{-\alpha t}) + B * (e^{-\beta t}) \quad (1)$$

$$C(t) = \frac{DK01}{V(K01 - K10)} (e^{-K10t} - e^{-K01t}) \quad (2)$$

Equation 1 was used to predict the deslorelin degradation in the membrane fraction and cytosolic fractions. Eq. 2 was used to predict the concentrations of deslorelin degradation products.

RESULTS

Deslorelin Degradation

Deslorelin was degraded in the membrane and cytosolic fractions of Calu-1 cells. Deslorelin degradation in the membrane and cytosolic fractions could be fitted to two-compartment models with bolus input and reliable estimates of the degradation rate constants could be obtained. A plot of the observed and predicted concentrations of deslorelin remaining in the membrane and cytosolic fractions at various incubation times is shown in Fig. 2. The correlations (r^2) between the observed and the predicted deslorelin concentrations were 0.994 and 0.997 in the membrane and cytosolic fractions, respectively. The deslorelin degradation was more rapid in the cytosolic fraction compared to the membrane fraction. At the end of one hour of incubation, 12 and 70% deslorelin remained in the cytosolic and membrane fractions, respectively. The initial first-order degradation rate constant (α) for deslorelin in the membrane fraction was 0.48 h^{-1} (SE 0.0026) and the terminal degradation rate constant was 0.0155 h^{-1} (SE 0.000067). The deslorelin degradation in the cytosolic fraction was more rapid with the α and β values being 3.45 h^{-1} (SE 0.02) and 0.1437 h^{-1} (SE 0.005), respectively. Table III (see later) summarizes the parameter estimations for deslorelin degradation in the cytosolic and membrane fractions.

Typical HPLC chromatograms of deslorelin along with its degradation products at the end of 6 h of incubation in the membrane and cytosolic fractions are shown in Fig. 3. Whereas three major peaks with retention times at 16, 22, and 23 min were detected in the membrane fraction (Fig. 3a), in the cytosolic fraction, an additional peak was observed at 11 min (Fig. 3b). The amino acid analysis and the mass spectrometric data for the analysis of the degradation products are presented in Tables I & II. The structural assignment of the peptide degradation products was done as reported by Brudel

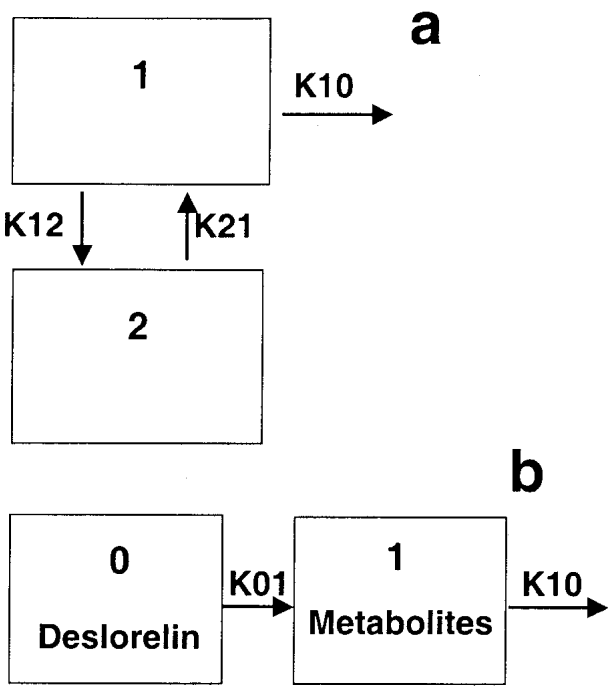


Fig. 1. Models used to fit deslorelin degradation (a) and deslorelin degradation product time course (b) in the membrane and cytosolic fractions. K01 and K10 are first order rate constants and the lag times in all cases were assumed to be zero.

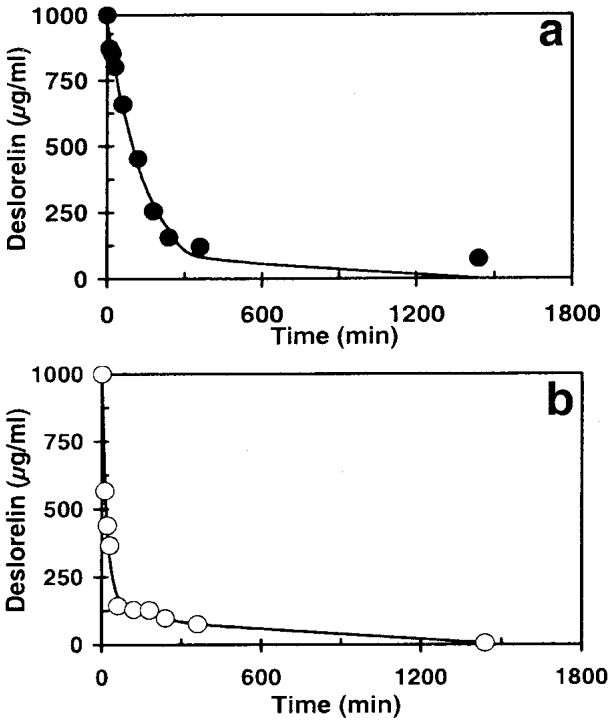


Fig. 2. Plots of deslorelin remaining vs. time in the membrane fraction (a) and the cytsolic fraction (b) of Calu-1 cells. Data points represent mean ± standard deviations of observed concentrations for n = 3 and the line represents concentrations predicted using pharmacokinetic models. Wherever invisible, the error bars are smaller than the symbols.

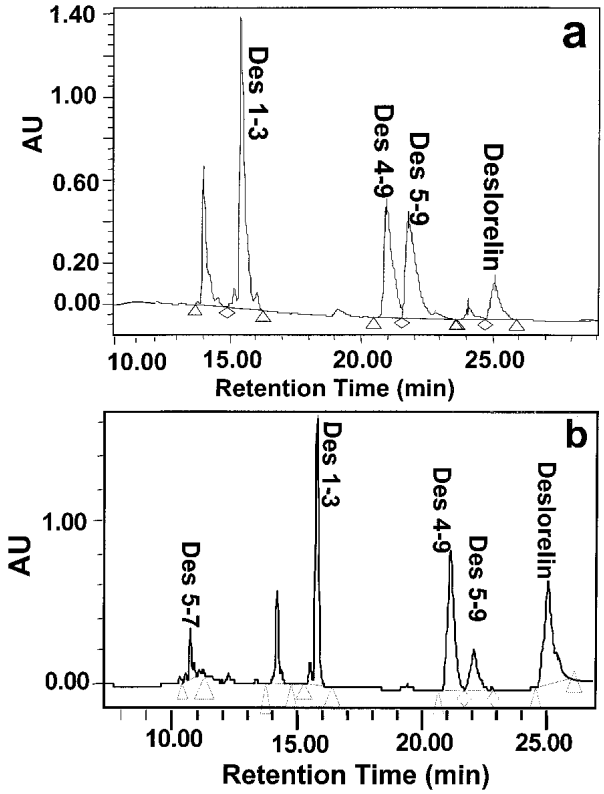


Fig. 3. Typical chromatograms showing the degradation products of deslorelin at the end of 6 hours incubation in the membrane fraction (a) and the cytosolic fraction (b) of Calu-1 cells.

et al. (1994). This analysis indicated that the N-terminal tripeptide pGlu-His-Trp- (Des1-3) eluted at 16 min and the C-terminal hexa- and penta-peptides, -Ser-Tyr-D-Trp-Leu-Arg-ProNHEt (Des4-9) and Tyr-D-Trp-Leu-Arg-ProNHEt (Des5-9), eluted at 22 and 23 min, respectively. The small peak at 11 min in the cytosolic fraction corresponded to the Des5-7 fraction containing Tyr-D-Trp-Leu. This low intensity peak was observed only at 6 h and not at earlier time points indicating a slow formation, in low quantities, with a lag-time.

Kinetics of Deslorelin Degradation Products

The major deslorelin degradation products were quantified using pure synthesized degradation products. The concentration vs. time profile of the deslorelin degradation products Des1-3, Des4-9 and Des5-9 were fitted to a one-compartment absorption model with no lag time and first order elimination (Fig. 1b). Figure 4 shows the observed and the predicted concentrations of the major degradation products, Des1-3, Des4-9, and Des5-9 at various time points in the membrane and cytosolic fractions of Calu-1 cells. The best estimates for the rate constants of formation (K01) and elimination (K10) of the different degradation products were determined (Table III). The correlations (r^2) between the observed and predicted concentrations of Des1-3 and Des4-9 were >0.95, possibly because they formed in a single step and because they were not degraded within the first 6 h (Fig. 4). In the case of Des5-9, reliable estimates of the rate constants could not be obtained. Because only minute amounts (~0.052% initial drug peak area) of Des5-7 were formed at the end of 6 h, it was not quantified. The mass balance of the percentage of amount of deslorelin remaining and the amounts of Des1-3, Des4-9, and Des5-9 formed at various time points is shown in Fig. 5. Whereas 95% of the total drug could be accounted for in the membrane fraction, only 75% could be accounted for in the cytosolic fraction.

Degradation Pattern in the Membrane and Cytosolic Fractions

The rate constants for the degradation products obtained using the pharmacokinetic models are shown in Table III. The rate constant assignments were made with the assumption that the metabolites exhibit flip-flop kinetics, with the formation rate constant being slower than their elimination rate constant. This, the authors assumed on the basis that the fragments, which are unprotected at either N- or C- terminal are more likely to undergo degradation compared to the parent compound. This assumption however, has not been experimentally verified. Therefore, the possibility of normal kinetics cannot be ruled out. It should be noted, however, that the proposed model accurately predicted the profiles of the primary degradation products (Figs. 4a and 4b). With the pharmacokinetic models used, the authors were unable to fit Des5-9 levels reliably. This may be because the dose of Des4-9 that is available for the formation of this secondary metabolite could not be adequately input into the model.

Table I. Mass Spectrometric Analysis for the Identification of Deslorelin Degradation Products

Retention time (mins)	M_r (calc)[a]	m/z (MH^+)[b]	Sequence assignment	Deslorelin degradation product
16	452.3	453.3	pGlu-His-Trp	Des1-3
21	847.5	848.5	-Ser-Tyr-D-Trp-Leu-Arg-ProNHEt	Des4-9
22	761.4	762.4	-Tyr-D-Trp-Leu-Arg-ProNHEt	Des5-9
11[c]	353.2	354.2	-Tyr-D-Trp-Leu-	Des5-7
26	1281.5	1282.5	Intact deslorelin	Des1-9

[a] Monoisotopic masses.
[b] m/z values for the [MH^+] ions in the ESI mass spectra.
[c] Detected only in the cytosolic fraction.

Table II. Amino Acid Analysis for the Different Deslorelin Degradation Products Isolated by HPLC

Retention time (min)	Glu	His	Trp	Ser	Tyr	D-Trp	Leu	Arg	Pro
Membrane fraction									
16	4.17	4.11	[a]						
21				2.03	2.32		2.35	2.34	0.85
22					2.45		2.87	2.64	1.97
Cytosolic fraction									
16	3.09	2.50							
21				1.72	2.02		2.05	2.05	1.75
22					2.33		2.37	2.34	1.87
11					1.01		2.05		

[a] Tryptophan is hydrolysed and not determined in amino acid analysis. Results reported have been confirmed with peptide sequencing.

Effect of Enzyme Inhibitors on Deslorelin Degradation

The effect of different enzyme inhibitors on the degradation of deslorelin in the membrane and cytosolic fractions of Calu-1 cells was evaluated. Disodium EDTA inhibited deslorelin degradation and increased the percent deslorelin remaining from 42 ± 5.6 to 61.8 ± 8.6% in the membrane fraction and from 28 ± 2.5 to 75 ± 3.8% in the cytosolic fraction. With captopril and phosphoramidon, the percent deslorelin remaining was 61 ± 2.19 and 37.8 ± 4.5% in the membrane fraction and 44 ± 1 and 25 ± 2.5% in the cytosolic fraction, respectively (Fig. 6). With the use of EDTA, the formation of all the degradation products was inhibited and the amount of Des1-3, Des4-9, and Des5-9 formed at the end of 6 h was reduced by 66.6 ± 3.56, 70 ± 6.33 and 85 ± 7.87% in the membrane fraction and by 80 ± 2.45, 75 ± 8.9 and 90 ± 3.65% in the cytosolic fraction, respectively, compared to controls. Captopril decreased the amount of Des1-3, Des4-9, and Des 5-9 by 32.25 ± 4.78, 25.5 ± 3.76, and 30.3 ± 3.765% in the membrane fraction and by 16.42 ± 3.2, 20.42 ± 5.1, 39.25 ± 4.34% in the cytosolic fractions, respectively. In the case of phosphoramidon, the amount of Des1-3 decreased by 43.4 ± 4.4 and 15 ± 5.8% in the membrane and cytosolic fractions, respectively. However under our HPLC conditions, phosphoramidon eluted at 21 min, interfering with the quantification of Des4-9 and Des5-9 in phosphoramidon studies. Table IV summarizes the effect of enzyme inhibitors on the concentration of the deslorelin at the end of 6 h in the membrane and cytosolic fractions.

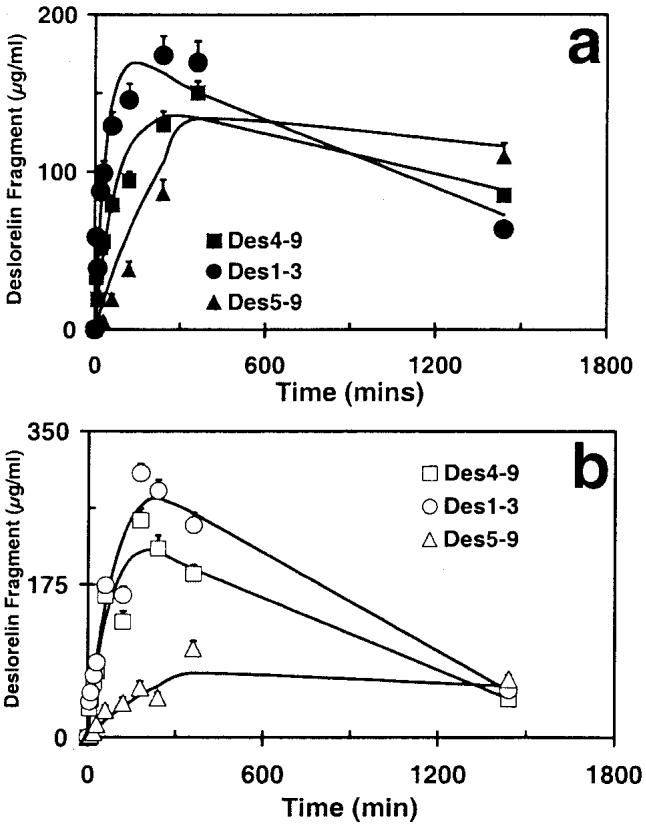


Fig. 4. Time course of Des 1–3, Des 4–9, and Des 5–9 in the membrane (a) and cytosolic (b) fraction of Calu-1 cells. Data points represent mean ± standard deviations of observed concentrations for n = 3 and the lines represent concentrations predicted using pharmacokinetic models.

DISCUSSION

Based on the degradation products formed, three cleavage sites are likely in deslorelin: 1) bond between Trp^3-Ser^4, 2) bond between Ser^4-Tyr^5, and 3) bond between Leu^7-Arg^8. Whereas cleavages 1 and 2 occur in membrane and cytosolic fractions, cleavage 3 was observed only in the cytosolic fraction. These degradation sites indicate the involvement of endopeptidases in deslorelin degradation. Three membrane bound endopeptidases, EP 24.15, EP 24.11, and angiotensin converting enzyme (ACE) have been shown to cleave LHRH at Tyr^5-Gly^6, Gly^6-Leu^7, Trp^3-Ser^4, and the Leu^7-Arg^8 bonds, respectively (16). From the major degradation products formed and the time course of formation of these products, the proposed pathways of deslorelin degradation in the membrane and cytosolic fractions of Calu-1 cells are shown in Scheme I.

The amount of Des5-9 formed in the membrane fraction was greater compared to the cytosolic fraction. In the cytosolic fraction, the enzymes for the formation of this fragment may be less abundant or the enzymes for the degradation of this fragment may be more abundant. Indeed, the appearance of Des5-7 exclusively in the cytosolic fraction leads the au-

Table III. The Degradation Rate Constants of Deslorelin and the Formation and Degradation Rate Constants of Deslorelin Degradation in the Membrane and Cytosolic Fractions of Calu-1 Cells

	Rate constants for degradation/formation h^{-1}			
	Membrane fraction		Cytosolic fraction	
Product	K01 (CV%)	K10/α/β (CV%)	K01 (CV%)	K10/α/β (CV%)
Deslorelin		K10: 039 (10.2) α: 0.48 (0.94) β: 0.0155 (7.2)		K10: 0.666 (2.14) α: 3.45 (0.83)[a] β: 0.144 (3.77)[a]
Des1-3	0.0238 (5.89)	K10: 0.768 (3.52)	0.078 (2.35)	K10: 0.835 (2.99)
Des4-9	0.04 (3.47)	K10: 1.538 (2.44)	0.091 (2.51)	K10: 0.52 (2.11)
Des5-9	0.085 (495)	K10: 0.084 (429)	0.088 (1847)	K10: 0.09 (1833)

[a] Indicates significant difference at $p < 0.05$ compared to the corresponding rate constant in the membrane fraction.

thors to believe that the subsequent metabolism of Des5-9 is rapid in the cytosol. Whereas the sum of the deslorelin remaining and the Des1-3, Des4-9, and Des5-9 formed accounted for 95% of the deslorelin in the membrane fraction, it accounted for only 75% of total deslorelin in the cytosol (Fig. 5). This may be due to the formation of additional degradation products that were not detected in this study. Previous studies suggest that intracellular degradation is not important as it occurs only after LHRH analogs exerted their action at the membrane level (17). However, internalization of intact peptide and its maintenance within the cell is important for deslorelin because intracellular presence of intact form after binding to receptor is required for its therapeutic effect (18). In addition, for systemic delivery of a peptide following cellular entry, the peptide has to survive the intracellular degradation. The authors observed that the deslorelin half-life ($t_{1/2\ \alpha}$) was 100.52 and 12.5 min in the membrane and the cytosolic fractions, respectively. The higher degradation of deslorelin in the cytosolic fraction is consistent with the 100-fold higher abundance of enzymes in the cytosol (19). Cytosol contains a number of enzymes including pyroglutamate aminopeptidase (EC 3.4.19.3), post-proline-cleaving enzyme (EC 3.4. 21.26), and particle-bound gonadoliberin-degrading enzyme (EC3.4.99) that are capable of degrading LHRH analogs. The degradation half-life of LHRH in alveolar macrophages and Type II lung pneumocytes was 18.1 and 24.4 min, respectively, when the incubation mixtures contained 1.25 mg/ml protein content (20). Also, in rabbit nasal homogenates, the degradation half-life of LHRH was 19.7 min (21). Although a comparison between tissues and different cell culture models for degradation of a drug does not reflect a complete picture as a number of factors including species and culture conditions play a role, it can be seen that deslorelin is more stable than LHRH. In a previous study with bovine muscle tissue the authors demonstrated that deslorelin is more stable toward enzymatic degradation compared to native LHRH (22).

Previously it was shown that the higher activity of the synthetic LHRH analogs is due to their increased stability toward membrane bound endopeptidases (23). Although the

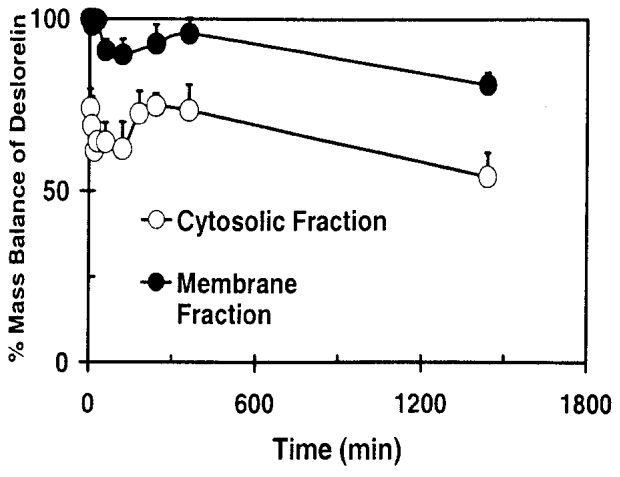


Fig. 5. Mass balance of deslorelin in the membrane and cytosolic fractions. Sum of deslorelin remaining, Des1–3, Des4–9, and Des 5–9 formed at various time points in the membrane and cytosolic fractions of Calu-1 cells is expressed as a percentage of initial deslorelin amount. Data is presented as mean ± standard deviation for n = 3.

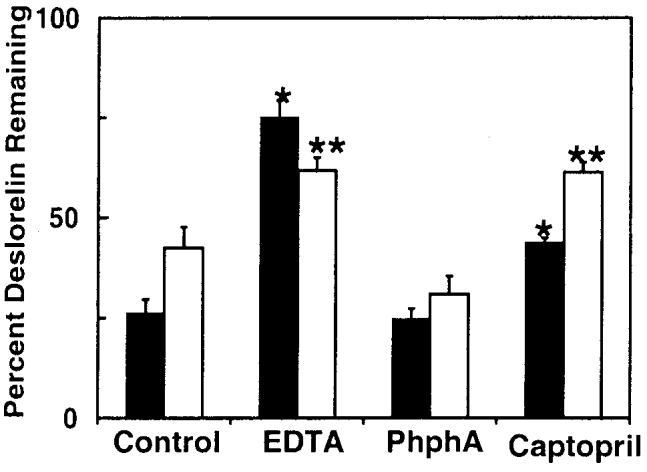


Fig. 6. Effect of EDTA, phosphoramidon (PhphA) and catopril on the precent deslorelin remaining at the end of 6 hours in the membrane (open bars) and cytosolic (closed bars) fractions of Calu-1 cells. * Indicates significant difference at $p < 0.05$ compared to control in the cytosolic fraction. ** Indicates significant difference at $p < 0.05$ compared to control in membrane fraction.

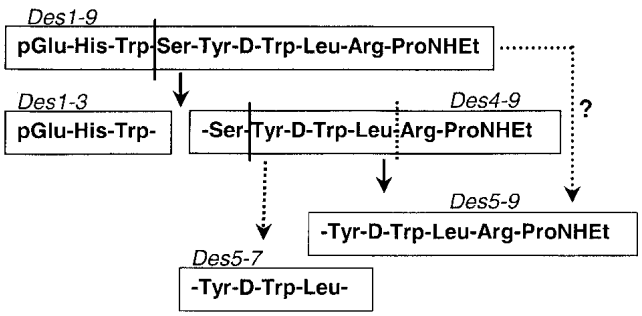
Table IV. Effect of Enzyme Inhibitors on the Concentration (μg/ml) of Deslorelin Degradation Products Formed at the End of 6 Hours in the Membrane and Cytosolic Fractions of Calu-1 Cells

Degradation product	Control	EDTA	Captopril	Phosphoramidon
Membrane fraction				
Des 1-3	98.6 ± 4.8	32.418 ± 3.3[a]	66.418 ± 5.63[a]	55.418 ± 4.6[a] [b]
Des4-9	89.6 ± 3.9	19.397 ± 5.0[a]	64.147 ± 5.5[a]	
Des5-9	185.6 ± 4.6	99.9 ± 4.9[a]	155.3 ± 4.8[a]	
Cytosolic fraction				
Des1-3	109.54 ± 6.7	29.29 ± 3.9[a]	93.54 ± 5.9[a]	94.29 ± 9.8
Des4-9	108.05 ± 2.0	32.8 ± 4.0[a]	87.55 ± 4.7[a]	
Des5-9	97.81 ± 3.3	7.56 ± 3.9[a]	58.56 ± 4.8[a]	

Data is expressed as mean ± SD, n = 4.
[a] Indicates $p < 0.05$ compared to corresponding control for each fragment.
[b] These products could not be separated in the presence of phosphoramidon.

D-Trp[6] in deslorelin makes it considerably stable toward degradation compared to native LHRH, this substitution alone is not sufficient to effectively block all degradation. It has been shown that at the time comparing the degradability of superactive analogs of LHRH by the membrane proteases, the order of degradability is: LHRH >> D-Phe[6]-LHRH >> D-Trp[3]-DPhe[6]-LHRH (23). Our studies also indicate that the Trp[3]-Ser[4] bond is a major site of enzymatic cleavage in deslorelin and a modification at the Trp[3] may be beneficial. Analogs with D-amino acid substitution at position 3 are not biologically active (23), indicating that whereas substitution of an unnatural amino acid at position 3 improves the stability, it is not a valid alternative to synthesize new analogs.

To elucidate involvement of different enzymes in deslorelin degradation, we studied the effect of enzyme inhibitors. Addition of disodium EDTA to the incubation mixture significantly inhibited deslorelin degradation and the formation of all the degradation products. This is consistent with the involvement of metallo-endopeptidases in the degradation of deslorelin. EDTA inactivates metallo-endopeptidases by a S_n1 type mechanism wherein the Zn ions from the active site spontaneously dissociate from the metalloenzyme and rapidly react with EDTA in solution (24). Because ACE is also a zinc-metallopeptidase that can be inhibited by EDTA, this may be another reason for the significant protection observed with EDTA (25). Indeed, following the addition of 2% EDTA, the intact deslorelin remaining after incubation with nasal, corneal, and conjunctival tissues was elevated by 113, 44, and 67%, respectively (9–11).



Scheme 1. Proposed degradation pathways of deslorelin in the membrane and cytosolic fractions of Calu-1 cells. Solid lines denote degradation in both membranes and cytosolic fractions whereas dashed lines denote degradation exclusive to the cytosolic fraction.

Use of an angiotensin converting enzyme inhibitor, captopril (30 μM), prevented the Trp[3]-Ser[4] bond cleavage in LHRH and inhibited the formation of LHRH 4-10 fragment in type II and A549 cell preparations (20). Conflicting evidence in another study revealed that captopril at 0.01 mM, 10 times the concentration required for the complete inhibition of ACE (26), was ineffective in decreasing the LHRH degradation in the membrane fractions of the anterior pituitary membrane (27). In our studies with Calu-1 cells, we found that captopril (0.1 mM) decreased the degradation of deslorelin and increased the percent remaining of deslorelin by 30% and 40% in the membrane and cytosolic fraction, respectively. However, the formation of Des4-9 and Des1-3 fragments was not completely abolished by captopril, indicating that ACE is only partially responsible for this cleavage. Because this is the first study to investigate the degradation pathways of an LHRH agonist in Calu-1 cells, the abundance of different enzymes in the different cell fractions of these cells is unknown. The protection offered by captopril in the cytosolic and membrane fractions indicates, however, that ACE is likely present in these fractions.

Apart from endopeptidase 24.11 and endopeptidase 24.15, another metalloendopeptidase, EC 3.4.24.18 (meprin), has been identified. Meprins are zinc-metalloproteases of the astacin family and of the metzincin family and cleave a range of biologically active peptides. Although, meprins are mostly expressed in the kidney brush border and in the intestine, there is also evidence for their expression in the nasal cavity (28). Endopeptidase-24.18 hydrolyzes bonds adjacent to aromatic or hydrophobic residues, which may include the Trp[3]-Ser[4] and the Ser[4]-Tyr[5] bonds in deslorelin.

Phosphoramidon, an inhibitor of endopeptidase 24.11 (29), did not offer any protection to deslorelin suggesting that EP 24.11 may not be involved in deslorelin degradation. Also, our previous studies showed that phosphoramidon and TPCK do not inhibit deslorelin degradation in the intact nasal, corneal and conjunctival tissues, indicating the involvement of peptidases insensitive to these inhibitors (9–11).

It has been shown that there may be an involvement of a phosphoramidon insensitive endopeptidase-2 (29) in the hydrolysis of peptide bonds 3-4 and 4-5 of buserelin, another LHRH analogue. This endopeptidase-2 is distinct from endopeptidase 24.11 in that it is insensitive to inhibition by phosphoramidon. It has zinc in its active site, has optimum activity at pH 7.3, and is inhibited by EDTA (30). Furthermore, endopeptidase-2 hydrolyzes LHRH primarily at the Trp^3-Ser^4 bond and can cleave the Ser^4-Tyr^5 and Tyr^5-Gly^6 bonds.

CONCLUSIONS

Deslorelin is degraded in the membrane and cytosolic fractions of Calu-1 cells. The primary sites of cleavage are the Trp^3-Ser^4 and Ser^4-Tyr^5 bonds. The degradation rate constants of deslorelin as well as Des1-3, and Des4-9 were higher in the cytosol compared to the membrane suggesting the greater abundance and variety of enzymes in this fraction. EDTA, a metallopeptidase inhibitor and captopril an ACE inhibitor, decreased deslorelin degradation by 20 and 22% in the membrane and 47 and 16% in the cytosol, respectively. This suggests the involvement of these enzymes in deslorelin degradation. The kinetics of deslorelin degradation and formation of the primary deslorelin degradation products could be described using simple pharmacokinetic models. However, for the secondary degradation products a more complex model, which takes into account the sequential metabolite formations, needs to be developed.

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