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RESEARCH PAPER

Inhibition of Corneal Metabolism of Deslorelin by EDTA and $ZnCl_2$

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ABSTRACT

It was the aim of this study to determine whether deslorelin is degraded by the rabbit corneal tissue and to further delineate the mechanisms. Deslorelin was incubated with intact cornea either alone or in the presence of 0.1 mM ouabain, 0.1% 2,4-dinitrophenol, 0.1 mM phosphoramidon, 0.1 mM N-tosyl-L-phenylalanine chloromethylketone (TPCK), 0.1-2% EDTA, 0.1-1% $ZnCl_2$, 0.1% dithiothreitol (DTT), or 0.1% N-ethylmaleimide (NEM) at 37°C. In addition, deslorelin alone was incubated with cornea at 4°C. Following a 90-min incubation, the supernatants were analyzed using a reversed-phase HPLC. Metabolite peaks observed in controls at 37°C were not detected in the low-temperature study, suggesting inhibition of metabolism at low temperature. Intact drug remaining in the supernatant was not altered by ouabain and dinitrophenol, suggesting that energy-dependent corneal uptake is not likely for deslorelin. Phosphoramidon and TPCK failed to alter deslorelin levels, indicating that phosphoramidon and TPCK-sensitive endopeptidases did not contribute to the observed metabolism. DTT and NEM also failed to affect deslorelin levels. However, 2% EDTA and 1% $ZnCl_2$ significantly elevated the intact deslorelin levels by 44 and 60%, respectively, and the metabolite peaks almost completely disappeared. These observations are consistent with the corneal metabolism of deslorelin by either metallo-peptidases or metal-dependent peptidases.

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INTRODUCTION

Deslorelin (MW = 1.3 kDa), a synthetic luteinizing hormone releasing hormone (LHRH) agonist, is 144-times more potent in vitro than the naturally occurring LHRH (1). The amino acid sequence of deslorelin is pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-NHEt. Deslorelin is of potential value in several disorders where LHRH analogs are indicated, including precocious puberty, fibroids, endometriosis, polycystic ovary disease, breast cancer, and prostate cancer (2,3). Due to the large molecular size and proteolytic degradation, peptide drugs such as deslorelin are poorly absorbed following oral administration (4). For this reason, several alternative routes including ocular route have been investigated for the delivery of peptide drugs. Indeed, the viability of ocular route for systemic delivery of LHRH has been demonstrated by Chiou and Chuang (5).

Despite better bioavailability of peptide drugs via ocular route when compared to the oral route, this route suffers from poor epithelial permeability, as do peptidases. Several reports suggested the ability of cornea and conjunctiva to degrade peptide drugs (6,7). Our earlier studies indicated that conjunctiva (8) as well as nasal tissue (9) are capable of degrading deslorelin. Prior to systemic drug absorption, as an eye drop comes in contact with cornea, conjunctiva, and nasal tissue (10), it was the objective of the current study to determine deslorelin metabolism in excised rabbit cornea and to further elucidate whether ouabain, 2,4-dinitrophenol, phosphoramidon, *N*-tosyl-L-phenylalanine chloromethylketone (TPCK), EDTA, $ZnCl_2$, dithiothreitol (DTT), and *N*-ethylmaleimide (NEM) can inhibit deslorelin metabolism. While ouabain and 2,4-dinitrophenol are known to inhibit active cellular transport of solutes (11,12), phosphoramidon has been shown to inhibit endopeptidase 24.11 (EC 3.4.24.11) that is capable of degrading LHRH at Ser^4-Tyr^5 bond (13). TPCK is known to inhibit an endopeptidase capable of degrading native LHRH at its Tyr^5-Gly^6 bond (14). EDTA, $ZnCl_2$, dithiothreitol, and *N*-ethylmaleimide are known to inhibit either metal-dependent peptidases or a metallo-endopeptidase (EC 3.424.15), which is known to degrade LHRH at Tyr^5-Gly^6 bond (15–17).

MATERIALS AND METHODS

Materials

From New Zealand white female rabbits, corneas were isolated and used within an hour after sacrifice. Deslorelin acetate (~99% purity) was a gift from Balance Pharmaceuticals, Inc. (Pacific Palisades, CA). Ouabain, 2,4-dinitrophenol, TPCK, phosphoramidon, DTT, NEM, $ZnCl_2$, NaH_2PO_4, and D-sorbitol were obtained from Sigma Chemical Co. (St. Louis, MO). Siliconized polypropylene microcentrifuge tubes (microtubes) and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ).

Solutions for Incubation

Deslorelin (1 mg/ml) solution was prepared with or without adjuvants in distilled water. The pH of all solutions was adjusted to 5.0 with either HCl or NaOH. The osmolalities of all solutions were adjusted to 300 mOsm/kg H_2O using D-sorbitol. In all studies except the low-temperature study, incubation solutions pre-warmed to 37°C were used. In the low-temperature study, the incubation mixture was used at 4°C. The microtubes containing the tissue and the incubation mixture were maintained at either 37 or 4°C in a water bath. The adjuvants tested were 0.1 mM ouabain, 0.1% 2,4-dinitrophenol, 0.1 mM phosphoramidon, 0.1 mM TPCK, 0.1–2% EDTA, 0.1–1% $ZnCl_2$, 0.1% DTT, and 0.1% NEM.

Corneal Isolation and Incubation

Rabbit eyes were isolated according to the procedure described by Kompella et al. (18). The cornea was excised, rinsed in 1.17% KCl, and gently blotted with a tissue paper. Subsequently, 50 mg of corneal tissue was weighed into each empty siliconized polypropylene microcentrifuge tube. To the corneal tissue thus obtained, 100 µl of an incubation mixture at the appropriate temperature was added. The microtube was gently vortexed and the preparation was maintained at 37 or 4°C for 90 min in a water bath. At the end of 90 min, the reaction was stopped by adding 900 µl of acetonitrile and water mixture (50:50 by volume) to the incubation mixture and by immediately placing the vial on ice. Subsequently, the samples were centrifuged at 4°C and 1000 × *g* for 10 min and the supernatants were analyzed using HPLC.

HPLC Assay

Deslorelin was quantified using a Shimadzu HPLC fitted with a Microsorb-MV 5 µm porous C-18 column (Rainin Instrument Company, Inc., Woburn, MA) as per the procedure described earlier (9). The HPLC system consisted of a SCL-6B system controller, one LC-

6A pump, a SIL-6B autoinjector, a SPD-6A UV spectrophotometric detector, and a CR-501 data processor. The mobile phase was composed of a 0.1 M phosphate buffer (pH 5.0)–acetonitrile mixture (70:30 volume). The flow rate was 1 ml/min and the detection wavelength was 220 nm. With this assay, the lowest detectable level of deslorelin was 1 μg/ml.

Data Analysis

Results are expressed as mean ± SD for $n = 3$ or more tissues obtained from 3 or more rabbits. Statistical significance of differences between two data points was assessed by unpaired Student's t-test. A p value of 0.05 or less was considered significant.

RESULTS

As can be seen in Fig. 1, while incubation solution devoid of deslorelin produced a peak at ~3.0 min, deslorelin eluted at ~7.3 min. Corneal tissue, when incubated with the vehicle devoid of deslorelin for 90 min at 37°C, yielded a peak at ~3.1 min [Fig. 1(b)], suggesting no interference between the intact deslorelin and the tissue-related peak under the HPLC conditions used. Furthermore, there was no interference between any of the adjuvant peaks and the intact desiorelin peak. Incubation of deslorelin with the corneal tissue for 90 min at 37°C yielded additional peaks apart from deslorelin peak and the corneal tissue peak [Fig. 1(c)]. The metabolite peaks completely disappeared following low-temperature incubation [Fig. 1(d)], but not following incubation with ouabain, dinitrophenol, phosphoramidon, TPCK, DTT, or NEM at 37°C (chromatograms not shown). On the other hand, in the presence of EDTA [Fig. 1(e)] and $ZnCl_2$, [Fig. 1(f)], the metabolite peaks almost completely disappeared.

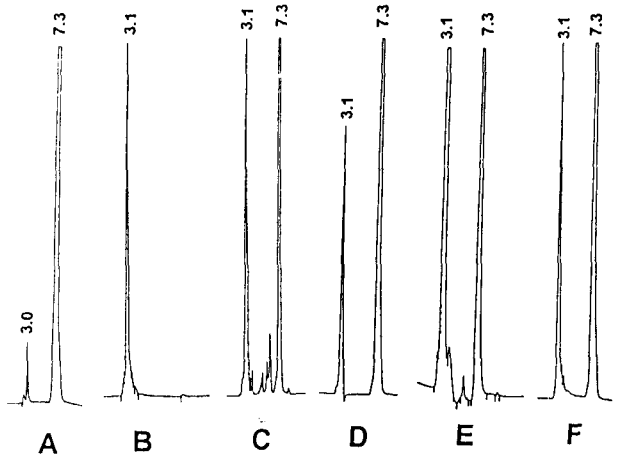

Figure 1. HPLC chromatograms of deslorelin. Key: (a) deslorelin control in the vehicle; (b) corneal tissue control; (c) deslorelin incubation with cornea at 37°C; (d) deslorelin incubation with cornea at 4°C; (e) deslorelin plus 2% EDTA incubation with cornea at 37°C; (f) deslorelin plus 1% $ZnCl_2$ incubation with cornea at 37°C. Retention time of deslorelin was ~7.3 min.

In controls, where deslorelin was incubated in the absence of cornea, 95 ± 9 μg deslorelin remained in the supernatants at the end of a 90-min incubation of 100 μg of deslorelin at 37°C. When deslorelin was incubated with cornea in the absence of any adjuvants, 47 ± 12 μg of deslorelin remained in the supernatants at the end of incubation. Figure 2 shows the effect of various inhibitors of cellular energy-dependent processes on the disappearance of deslorelin from supernatants. Although ouabain increased intact deslorelin levels by 31% from 47 ± 12 to 62 ± 3 μg, this response was not statistically significant. Furthermore, 45 ± 3 μg of deslorelin remaining in the presence of dinitrophenol was not different from controls. However, low-temperature (4°C) incubation of deslorelin with tissue significantly increased intact deslorelin levels by 51% to 71 ± 5 μg.

In the presence of TPCK and phosphoramidon, the intact deslorelin levels remaining at the end of 90 min were 59 ± 17 and 61 ± 4 μg, respectively (Fig. 3). These effects were not statistically significant. EDTA (2%), on the other hand, significantly increased intact deslorelin levels by 44% to 68 ± 2 μg. Furthermore, 1% $ZnCl_2$ increased deslorelin levels by 60% to 75 ± 13 μg ($p < 0.05$). Effects of 2% EDTA and 1% $ZnCl_2$ were not significantly different from the above low-temperature response observed. In the presence of 0.1% DTT and 0.1% NEM, deslorelin levels were 61 ± 4 and 54 ± 9 μg, respectively. These levels were not significantly different from controls.

In order to determine whether lower concentrations of EDTA can protect deslorelin from corneal metabolism, 0.1, 0.5, and 1% EDTA were also evaluated in corneal incubation studies. The results obtained are shown in Fig. 4. The deslorelin levels were changed by 23, 20, 49, and 44% to 58 ± 9, 56 ± 9, 70 ± 5, and 68 ± 2 μg, in the presence of 0.1, 0.5, 1, and 2% EDTA, respectively. Of all these concentrations, only 1 and 2% EDTA significantly elevated deslorelin levels. The responses to 1 and 2% EDTA were not statistically different.

Because 1% $ZnCl_2$ was effective in reducing deslorelin metabolism, lower concentrations were also

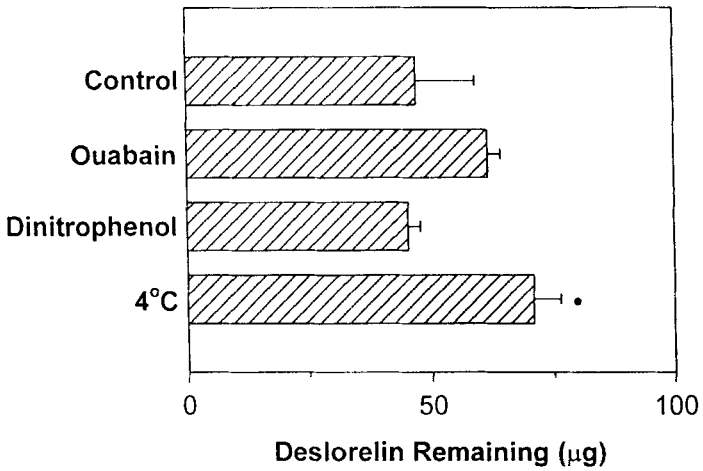

Figure 2. Effect of inhibitors of energy-dependent cellular processes on intact deslorelin levels remaining at 90 min following corneal tissue incubation. All studies, except 4°C study, were performed at 37°C. Ouabain and 2,4-dinitrophenol were used at a final concentration of 0.1 mM and 0.1%, respectively. Asterisk indicates a statistically significant difference from the control. Error bars denote SD for $n \geq 3$.

tested for their effects. Indeed, $ZnCl_2$ significantly elevated deslorelin levels at all the tested concentrations in the range, 0.1–1% (Fig. 5). It can be seen that deslorelin levels increased by 34, 72, and 60% to 63 ± 3, 81 ± 15, and 75 ± 13 μg, in the presence of 0.1, 0.5, and 1% $ZnCl_2$, respectively. Although both 0.5 and 1% $ZnCl_2$ were significantly more effective than 0.1% $ZnCl_2$, there was no significant difference between the effects of 0.5 and 1% $ZnCl_2$.

DISCUSSION

The findings of the current study show that deslorelin is metabolized by excised rabbit corneal tissue [Fig.

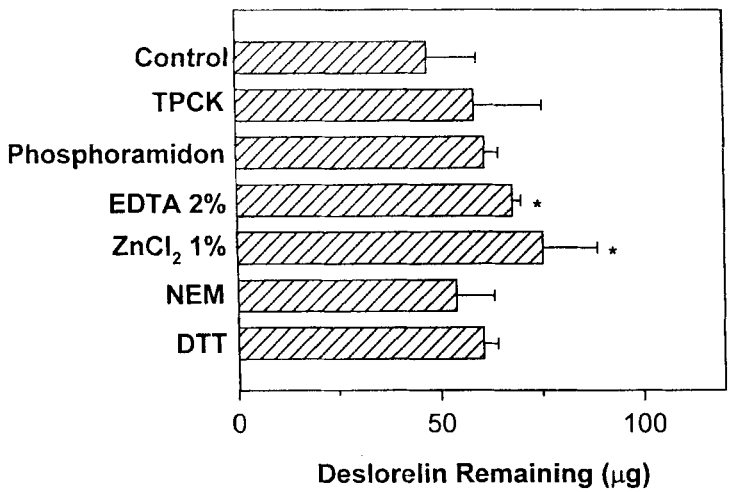

Figure 3. Effect of peptidase inhibitors on intact deslorelin levels remaining at 90 min following corneal tissue incubation. TPCK, phosphoramidon, EDTA, $ZnCl_2$, NEM, and DTT were used at a final concentration of 0.1 mM, 0.1 mM, 2%, 1%, 0.1%, and 1%, respectively. Asterisk indicates a statistically significant difference from the control. Error bars denote SD for $n \geq 3$.

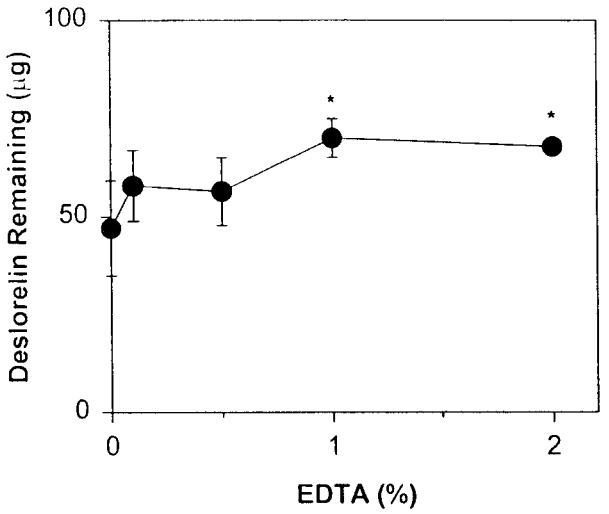

Figure 4. Effect of EDTA on intact deslorelin levels remaining at 90 min following corneal tissue incubation. EDTA was used at a final concentration of 0, 0.1, 0.5, 1, or 2%. Asterisk indicates a statistically significant difference from the control. Error bars denote SD for $n \geq 3$.

l(c)]. This metabolism could be inhibited at low temperature and in the presence of EDTA [Fig. 1(e)] and $ZnCl_2$ [Fig. 1(f)]. At 4°C, deslorelin levels remaining in the corneal tissue supernatants were increased by

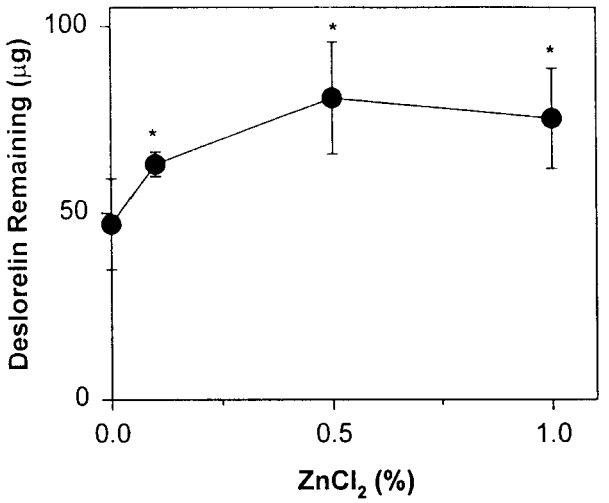

Figure 5. Effect of $ZnCl_2$ on intact deslorelin levels remaining at 90 min following corneal tissue incubation. $ZnCl_2$ was used at a final concentration of 0, 0.1, 0.5, or 1%. Asterisk indicates a statistically significant difference from the control. Error bars denote SD for $n \geq 3$.

51% to 71 μg and the metabolite peaks were abolished [(Fig. 1(d) and Fig. 2)], suggesting that the observed metabolism is temperature-dependent. In the presence of ouabain as well as dinitrophenol, deslorelin levels in the supernatants were not significantly increased (Fig. 2), suggesting that cellular uptake did not contribute to the disappearance of deslorelin observed in the current study.

Because endopeptidase 24.11, an enzyme capable of cleaving LHRH at the Ser^4-Tyr^5 bond, can be inhibited by phosphoramidon (13), lack of effect of phosphoramidon on the intact deslorelin levels in tissue supernatants (Fig. 3) suggests that endopeptidase 24.11 is not responsible for the observed deslorelin metabolism. Advis et al. (14) demonstrated that TPCK inhibited an endopeptidase that cleaved LHRH at the Tyr^5-Gly^6 bond. This bond in deslorelin is replaced by Tyr^5-D-Trp^6. Possibly due to the proteolytic resistance offered by the D-amino acid substitution, TPCK did not further protect deslorelin from corneal degradation (Fig. 3).

From the observations made in this study, it appears that EDTA-sensitive metabolism is probably the primary event in the observed metabolism, as 2% EDTA increased the deslorelin levels in supernatants by 44% to 68 μg (Fig. 3), and the metabolite peaks were almost completely abolished [Fig. l(e)]. No significant protection was offered by EDTA at 0.1 and 0.5% (Fig. 4). As EDTA is known to inhibit endopeptidase 24.15, which is a metallo-endopeptidase that degrades LHRH (15), the findings of the present study support the notion that deslorelin is susceptible to metabolism by a metallo-endopeptidase present in the plasma membrane of corneal epithelial cells. This observation is further supported by the inhibitory effects of 0.1–1% $ZnCl_2$ (Fig. 5). When used at 1% concentration, $ZnCl_2$ elevated deslorelin levels by 60% to 75 μg (Fig. 3) and abolished the metabolite peaks [Fig. 1(f)]. Similar metallo-endopeptidase inhibitory effects of EDTA and $ZnCl_2$ were reported for LHRH in the endothelial cells of ovine median eminence (16). Although zinc is at the active center and is essential for the activity of many metallo-enzymes, when present at high concentrations, zinc can inhibit the activity of metallo-enzymes. This is thought to occur as a result of conformational changes induced in the enzyme by high concentrations of zinc. In the median eminence, both dithiothreitol (a disulfide-reducing agent) and *N*-ethylmaleimide (a thiol-blocking agent) also inhibited LHRH metabolism. However, these two agents did not inhibit corneal metabolism of deslorelin (Fig. 3), suggesting that the exposed thiol groups of

membrane peptidases are not important for the observed metabolism of deslorelin. Tissue differences in the type and location of enzymes may also account for this difference. As the pGlu residue on the N-terminal is expected to make deslorelin resistant to most aminopeptidases and as the ethylamide residue on the carboxyterminal is expected to protect it from carboxypeptidases, we speculate that EDTA and $ZnCl_2$ inhibited a metallo-endopeptidases capable of degrading deslorelin. As EDTA and $ZnCl_2$ are capable of inhibiting EC 3.4.24.15, a metallo-endopeptidase which degrades deslorelin at the Tyr^5 -Gly^6 bond, it is not clear whether Tyr^5 -D-Trp^6 or some other bond in desiorelin was cleaved by this enzyme.

Within 90 min, as much as 53% (as 47 μg remained following corneal incubation) of deslorelin disappeared in the control corneal incubation study (Fig. 2). Of this, non-specific microtube binding accounts for 5% of the disappearance as 95 μg of deslorelin remained at the end of 90 min incubation of deslorelin solution (without inhibitors) in microtubes at 37°C. The remaining 48% can be attributed to corneal tissue binding and entrapment between cells, uptake, and metabolism. Energy-dependent tissue uptake appears not to contribute significantly to the disappearance of deslorelin. At 4°C, it can be anticipated that energy-dependent tissue uptake and metabolism are absent. The 71 μg remaining at the end of low-temperature study suggests that about 24% (95 μg remaining in the controls without tissue –71 μg remaining in the low-temperature study) of the drug is bound to the surface or entrapped between the epithelial cells. Thus, 29% of the initial drug used can be accounted for by microtube binding (5%), tissue binding and entrapment (24%), and tissue uptake (0%), Therefore, at least 24% (53% disappearance in corneal control minus 29%) of the drug appears to have been metabolized in the current study. It is likely that the membrane-bound/entrapped fraction (16%) may also have undergone extensive metabolism at 37°C.

Although the exact site of metabolism cannot be stated based on our observations, it is likely that deslorelin was metabolized by corneal membrane-bound enzymes rather than intracellular enzymes. This is primarily because deslorelin did not significantly enter the epithelial cells by active uptake mechanisms. Furthermore, by virtue of its size, it is unlikely that deslorelin significantly diffused into the cells. Thus, deslorelin entering the cells is insignificant, thereby limiting its degradation to those enzymes that are present on the corneal surface and the intercellular spaces.

In summary, the rabbit corneal tissue degraded deslorelin to the extent of 24% or more. The observed metabolism could be inhibited at 4°C and in the presence of EDTA or $ZnCl_2$. Thus, metallo-endopeptidase or metal-dependent peptidases appear to play a major role in deslorelin metabolism. Under the experimental conditions used, TPCK-sensitive endopeptidase and phosphoramidon-sensitive endopeptidase 24.11 did not play a role in deslorelin metabolism. Also cellular uptake and subsequent metabolism is unlikely to have contributed to deslorelin metabolism because ouabain and dinitrophenol, two agents capable of impairing active uptake of solutes by the cell, did not affect deslorelin levels.

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