Iontophoresis of Nafarelin Across Human Skin *in Vitro*

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INTRODUCTION

Due to proteolytic enzymes in the gastro-intestinal tract and extensive "first-pass" hepatic metabolism, proteins and peptides are not usually active following oral administration. However, other routes of drug delivery, including transdermal, provide the possibility of avoiding the hepato-gastrointestinal "first-pass" elimination and of better patient compliance (1). Because peptides and proteins have large molecular weights, are mostly hydrophilic and are often charged, their passive transdermal delivery is impossible, and an enhancement strategy is therefore essential. Iontophoresis, which employs an electrical potential gradient to drive substances through the skin, represents one approach to increase the skin's permeability (1–3).

Nafarelin is a decapeptide analog of leutinizing hormone releasing hormone (LHRH). It is a potent superagonist and has several physiological effects in both females and males (4, 5). The anodal iontophoresis of Nafarelin across both human and hairless mouse skin (hms) has been demonstrated (6, 7). Detailed study of its electrotransport through hms has revealed that the mechanism of enhanced delivery is primarily via electroosmosis; however, the peptide associates strongly with the skin's net negative charge, effectively neutralizing this property, and thereby inhibiting its own permeation (6–8). It has been shown that electroosmotic flow across hms (in the normal anodal-to-cathodal direction) can be reduced by a factor of ten due to the anodal iontophoresis of Nafarelin (8). The effect is dependent upon the concentration of Nafarelin in the donor chamber, becoming more important with increasing concentration (7).

In this paper, the iontophoresis of Nafarelin across human skin *in vitro* has been investigated, and a number of key issues, which impact upon the peptide's transport, has been examined: (a) the nature of the skin membrane used, (b) the applied current, (c) the donor concentration, and, briefly, (d) skin metabolism.

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MATERIALS & METHODS

Chemicals

Nafarelin ([D-Nal (2)^6]LHRH) acetate was a gift from Syntex Research (Palo Alto, CA). All other chemicals used were reagent grade or better.

Transport Measurements

The passive and iontophoretic transport of Nafarelin across human skin *in vitro* was measured in 'side-by-side' diffusion cells (LGA, Berkeley, CA) (6, 7). The tissue used to separate the donor (anode) and receptor (cathode) chambers was human skin (area = 0.64 cm^2) obtained either fresh post-surgically or from cadavers, within 24 hours of death. The skin was either from the abdomen or thigh. The permeation of the peptide was determined through either (a) epidermis + dermis (dermatomed full-thickness skin, 300–500 μm), or (b) epidermis alone (isolated by heat-separation or by exposure to ammonia vapor (9)), or (c) dermis alone.

The donor and receptor solutions were, respectively, 3.25 mL of Nafarelin acetate at various concentrations (see below) in 14 mM NaCl, and 3.25 mL of phosphate-buffered saline at pH 7. Under these circumstances, the donor solution pH was between 5 and 6, and the peptide carried an approximate net charge of +1 (6, 7); variation of Nafarelin concentration did not alter this situation significantly. The receptor chamber was magnetically stirred. To avoid electrochemical degradation of Nafarelin in the donor and receptor compartments, salt bridges (1 M NaCl in 3% w/w agar) were used to isolate the electrodes from the peptide solutions as previously described (6, 7). The electrodes were Ag/AgCl, prepared in the usual way (10).

In the iontophoretic experiments, constant current was delivered to the electrodes from a custom-built power supply (Professional Design and Development Services, Berkeley, CA) interfaced to a Macintosh IIfx computer (Apple Computer, Inc., Cupertino, CA). Current was passed for 24 hours. To assess Nafarelin delivery, 0.5 mL aliquots of the receptor phase were withdrawn every 2 hours (beginning at t = 4 hr) and analyzed by HPLC (6). Fresh buffer was used to replace each 0.5 mL aliquot withdrawn. After 24 hours of current passage, Nafarelin transport was monitored for a further 4–8 hours to observe desorption of the peptide from the skin. In all cases studied, the corresponding no-current controls were performed using identical protocols but without application of the electric field. All measurements were made in triplicate at least. Statistical comparisons utilized ANOVA and Student's t-test as appropriate.

Variables Examined

The parameters investigated in this study were:- (a) The nature of the skin membrane—transport was compared through epidermis + dermis, epidermis alone, and simply dermis; two procedures for preparing the epidermis were also examined. (b) The applied current—using a donor concentration of Nafarelin of 1 mg/mL, transport at 315 μA/cm^2 was compared to that at 630 μA/cm^2. (c) Nafarelin concentration—at 630 μA/cm^2, the effect of varying the initial peptide level in the donor compartment (0.1, 0.5, and 1.0 mg/mL, equivalent respectively to about

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0.07, 0.35, and 0.7 mM) was examined. (d) The HPLC chromatograms were evaluated qualitatively for peptide metabolism.

RESULTS AND DISCUSSION

It was possible to deliver Nafarelin across human epidermis *in vitro* by iontophoresis (Table I). Less peptide permeated full-thickness skin in the same time period, presumably reflecting a kinetic effect associated with the much longer diffusion pathlength through this much thicker piece of tissue. Similar to the results reported for hms (6), a reservoir of peptide was established in the epidermis, as indicated by the amounts desorbed from the membrane during the post-iontophoresis period. For example, at an applied concentration of 0.5 mg/mL and a current density of 0.315 mA/cm^2, 18.9 (±5.5) nmol/cm^2 were transported during 24 hours of iontophoresis; 2 hours later, during which time no current was passed, this quantity had increased to 20.3 (±5.5) nmol/cm^2, and by 4 hours post-iontophoresis, the cumulative peptide was 21.2 (±6.1) nmol/cm^2). Iontophoresis across isolated dermis, lacking the skin's naturally impermeable tissue layer, the epidermis, was facile as expected. Passively, although once again the dermis alone was penetrated by Nafarelin, skin possessing an intact epidermis proved very resistant to transport. Overall, these experiments confirmed (a) that the outermost layer of the skin represents the principal barrier to both passive and iontophoretic delivery, and (b) that *in vitro* diffusion cell experiments using full-thickness human skin, which lacks its natural blood supply, may underpredict iontophoretic delivery due to sequestration of the permeating molecule (11). For this reason, more sophisticated experimental models, such as the isolated perfused porcine skin flap (11–13), are likely to provide data more representative of *in vivo* electrotransport. Parenthetically, it should be noted that the epidermal isolation technique employed (heat separation versus exposure to ammonia vapor) did not significantly impact upon the cumulative quantity of peptide transported in 24 hours of iontophoresis.

The results in Table II show that increasing the applied current density improved proportionally, and significantly ($p < 0.05$), the quantity of peptide delivered across the epidermis, at all of the applied Nafarelin concentrations considered. These findings are consistent with previous observations for Nafarelin, LHRH and its analogs, and other peptides (6, 7, 12–15).

Further examination of Table II, however, reveals that the concentration dependence of Nafarelin delivery by iontophoresis (at both 'low' and 'high' current densities) is quite unusual. The Nafarelin concentration was varied between 0.1 and 1 mg/mL. Remarkably, Nafarelin transport increased with decreasing donor concentration. Figure 1 illustrates this phenomenon, at an applied current density of 0.63 mA/cm^2, over the 24-hour period of iontophoresis. The reason for this observation has been discussed in detail elsewhere (7). At high concentration, Nafarelin apparently binds strongly to the electronegative skin and dramatically decreases electroosmotic flow from the anode to cathode (6–8). Given the high concentration of ions present in the donor solutions in the experiments described here, it is likely that electroosmosis is the major mechanism of enhanced Nafarelin transport in the presence of the applied potential gradient. At the lowest concentration, on the other hand, binding of Nafarelin is less and the electroosmotic flow is not as seriously impeded as it is at high Nafarelin concentration. This results in the unexpected inverse dependence of transport on donor peptide concentration. A similar observation has been made in experiments using Leuprolide, another analog of LHRH (15).

Table I. Iontophoretic[a] Transport (mean ± SD) of Nafarelin[b] Across Human Skin Tissue *in Vitro*

Skin Membrane	Cumulative Amount of Nafarelin Delivered in 24 Hours (nmol/cm^2)	n[c]
Whole Skin	3.97 ± 0.1[d]	3
Epidermis	28.0 ± 13.3[e]	5
Dermis	125 ± 28.5[f]	6

[a] Constant current (0.63 mA/cm^2) applied for 24 hours.
[b] Initial donor (anodal) compartment concentration = 0.5 mg/mL.
[c] Number of replicates.
[d] Passive control showed no measurable transport of peptide.
[e] Passively, <2 nmol/cm^2 were transported.
[f] Without current, the delivery of Nafarelin was 20–30 nmol/cm^2.

Table II. Iontophoretic Transport (mean ± SD) of Nafarelin Across Human Skin Tissue *in Vitro* as a Function of Donor Concentration and Applied Current Density

Donor (Anodal) Concentration (mg/mL)	Cumulative Amount of Nafarelin Delivered in 24 Hours (nmol/cm^2)[a]		n[b]
	0.315 mA/cm^2	0.630 mA/cm^2	
0.1	27.3 ± 13.3	45.2 ± 5.3	5
0.5	18.9 ± 5.5	28.0 ± 13.3	5
1.0	5.95 ± 0.1	10.4 ± 1.9	6

[a] All passive controls were < 2 nmol/cm^2 and were most usually below the limit of detection of the HPLC assay.
[b] Number of replicates.

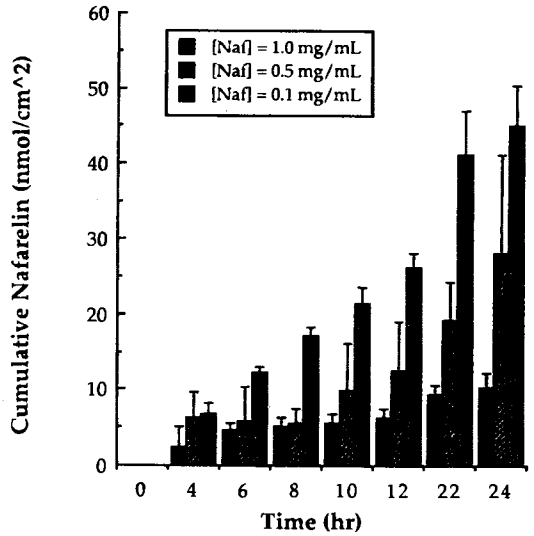

Fig. 1. Cumulative amount of Nafarelin delivered by iontophoresis at 0.63 mA/cm^2, as a function of time, across human epidermis *in vitro*. Data following application of the peptide at three different concentrations are shown. Average values (± standard deviation) for six replicates at each concentration and timepoint are presented.

Comparison of the data presented here with that obtained using hms, under comparable conditions (6, 7), (viz., peptide concentration in the donor phase ~1 mg/mL; constant current density ~0.4 mA/cm^2; 24 hours iontophoresis; HPLC analysis of permeated drug), reveals that Nafarelin fluxes across the two skin tissues are similar, on the order of 20 nmol/cm^2/hr (in other words, only a very small percentage of the applied Nafarelin is in fact transported across the skin). The average delivery rate, therefore, translates to ~1 μg/cm^2/hr and it is appropriate to ask whether this is a pharmacologically meaningful result. Given that the currently approved intranasal dosage of Nafarelin is 200 μg twice a day, with a typical bioavailability of about 3%, it follows that this formulation delivers 10–20 μg per day (16). Therefore, our iontophoretic fluxes are quite relevant, since an area of only 10–20 cm^2 would need to be covered in order that the target dose be delivered. Of course, this does not mean that one necessarily has a viable dosage form—however, it does argue that this research is pertinent to 'real-world' objectives.

Finally, a comment should be made on the metabolism of Nafarelin during its electrotransport across the skin. The HPLC analyses revealed that metabolites of the peptide were detectable in the receptor phase (at least 4 species were consistently found), and that control experiments indicated that the origin of this peptide breakdown was the skin itself (17). A greater percentage of the peptide was metabolized when the absolute flux measured was small, an outcome that has been reported previously for the non-peptide drug, nitroglycerin (18, 19). How important is the metabolism of Nafarelin observed? Clearly, the ultimate clinical delivery of the peptide via the skin would require a complete characterization of the metabolites and the extent to which each is formed. However, at the level of the *in vitro* studies used to measure electrotransport here, the ultimate relevance of the model must be questioned. In other words, are the profiles of metabolism and the nature and quantities of the metabolites formed in these experiments (even with the 'freshest' skin sample available) reflective of the *in vivo* situation? Unfortunately, the question is very difficult to answer because of the lack of an unambiguous method to measure skin metabolism *in vivo*. Thus, absolute validation of an *in vitro* methodology is impossible. Nevertheless, it is clear that additional, further effort must be directed at this question, and that innovative methodology should be developed; one might again look to the isolated perfused porcine skin flap (11–13) as a potential strategy with which to obtain meaningful results.

In conclusion, the results in this paper show that across human skin *in vitro:* (i) Iontophoretic Nafarelin delivery is rate-limited by the epidermis. (ii) Iontophoresis enhances the transdermal transport of Nafarelin (compared to passive penetration). (iii) The mechanism of enhanced transport of Nafarelin by iontophoresis is electroosmosis. (iv) The concentration dependence of iontophoretic transport is unexpected and suggests that the peptide binds strongly to the electronegative skin. (v) Metabolism of Nafarelin during its electrotransport can occur, and that further examination of this phenomenon is warranted.

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