Vaginal Absorption of Insulin in the Rat: Effect of Penetration Enhancers on Insulin Uptake and Mucosal Histology

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The absorption of insulin across the vaginal mucosa into the systemic circulation was studied in ovariectomized rats given subsequent estrogen treatment. Blood glucose levels were determined as an indirect measure of insulin absorption, and the effect of various enhancers on the hypoglycemic response was investigated. In the absence of any enhancer, no decrease in blood glucose levels was observed after vaginal administration of insulin. However, the coadministration of sodium taurodihydrofusidate, polyoxyethylene-9-lauryl ether, lysophosphatidylcholine, palmitoylcarnitine chloride, and lysophosphatidylglycerol significantly increased hypoglycemia, whereas citric acid had little effect. The histological changes in the vaginal epithelium after treatment with the enhancer systems were variable and often severe. While the efficacy of these compounds in promoting the vaginal absorption of insulin is encouraging, their mechanisms of action and long-term histological effects are yet to be defined.

KEY WORDS: vaginal administration; absorption enhancer; vaginal histology; insulin; rat.

INTRODUCTION

The vagina is a potential site for the systemic absorption of therapeutic peptides and proteins, particularly for those intended primarily for the treatment of female related diseases. The advantages and disadvantages of the vaginal route for peptide and protein drug delivery have been discussed previously (1). As at other mucosal sites, such as the nose, mouth, and rectum, the coadministration of absorption enhancing agents is normally necessary to achieve therapeutically relevant plasma levels of these large, hydrophilic compounds (2). Few papers have been published on the vaginal absorption of peptides and the effect of absorption enhancers on the vaginal epithelium.

The vaginal absorption of gentamicin, a model hydrophilic drug, was studied in ovariectomized rats using a variety of enhancers which displayed different physicochemical properties (3). The amphiphilic compounds, polyoxyethylene-9-lauryl ether (laureth-9), lysophosphatidylcholine (LPC), and palmitoylcarnitine chloride (PCC), were found to be highly effective absorption enhancers, but all induced epithelial damage. Citric acid, a chelating agent used to increase the vaginal absorption of a luteinizing hormone releasing hormone (LH-RH) analogue in rats (4), was found to be less effective. In the ovariectomized rat, the vaginal epithelium is thin and both vaginal absorption and drug-induced damage may be greater than in the normal female rat or in women. Therefore, an alternative rat model with a thicker vaginal epithelium was employed in the present study.

Insulin, which has been extensively studied for delivery via other transmucosal routes, was selected as a model peptide and coadministered with the range of enhancers used previously, (3) as well as sodium taurodihydrofusidate (STDHF) and lysophosphatidylglycerol (LPG). STDHF has been used to increase the nasal absorption of insulin and is thought to have considerable potential as an effective enhancer with minimal effects on mucosal histology (5). LPG is structurally similar to LPC and was investigated as a novel enhancing agent.

MATERIALS AND METHODS

Materials

17-β-Estradiol (Sigma Chemical Company Ltd., Dorset, U.K.) was prepared as a solution in arachis oil at a concentration of 100 μg/ml. Semisynthetic human sodium insulin was obtained as a gift from Novo-Nordisk (Denmark) and prepared as a solution in phosphate buffer, pH 7.3 to 7.4, at a concentration of 20 IU/ml. The water content of the insulin sample was determined by fluorometric analysis of the prepared solution. According to the product data sheet, a 1 mg/ml (28 IU/ml) insulin solution in a 1-cm^3 cuvette absorbs 1.058 at 276 nm. Thus, the water content was found to be 14% and the weight of insulin used was adjusted accordingly.

In some experiments, absorption enhancers were added separately to the insulin solutions at the following concentrations: 0.5% (w/v) L-α-lysophosphatidylcholine (LPC), 0.5% (w/v) L-α-lysophosphatidylglycerol (LPG), 1% (w/v) palmitoyl-DL-carnitine chloride (PCC), 1% (w/v) polyoxyethylene-9-lauryl ether (laureth-9), 10% (w/v) citric acid (all Sigma Chemical Company Ltd., Dorset, U.K.), and 1% (w/v) sodium taurodihydrofusidate (STDHF) (California Biotechnology). These enhancer concentrations were selected in accordance with published literature on mucosal delivery (3,5). All other chemicals used were of reagent grade.

Vaginal Administration of Insulin and Enhancers to Rats

Female Wistar rats (JABU, Sutton Bonington, U.K.) weighing approximately 200 g were bilaterally ovariectomized under halothane anaesthesia. The operation wounds were closed with Michel clips, which were removed after 10 days. The animals were allowed to recover for at least 2 weeks before receiving further treatment. Twenty-four hours prior to the drug absorption studies, 100 μl of estradiol solution (approximately 40 μg/kg) was administered by subcutaneous injection. The dose of estradiol and time of administration, relative to the absorption study, were chosen

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after preliminary studies showed that the vaginal epithelium of this rat model demonstrated consistent morphology and was thickened without signs of cornification.

After fasting overnight, groups of rats (n = 3–7) were anesthetized by intraperitoneal injection of 60 mg/kg pentobarbitone sodium (60 mg/ml, Sagatal, May and Baker). After tracheotomy and cannulation of the carotid artery and jugular vein to allow removal of blood samples and replacement of blood volume with saline, respectively, the rats were prepared for vaginal dosing as described previously (3). Initial blood samples (100 μl) were collected in fluoride oxalate tubes (Sterilin, Northern Media). Insulin solutions were instilled into the vaginal tract (8 IU/400 μl/kg) and blood samples were taken at intervals over 4 hr. As a control experiment, buffer solution alone (400 μl/kg) was administered vaginally to three rats and blood samples were collected as before. All samples were stored at 4°C prior to analysis within 4 hr. Blood glucose levels were determined by the glucose oxidase method using a Yellow Springs Instrument 23AM analyzer, calibrated for glucose measurement in the range 0 to 10 mmol/L. Blood glucose levels were expressed as a percentage of the basal concentration, which was calculated as the mean value of the three samples taken prior to drug administration.

The areas under the curves (AUCs) of blood glucose concentrations from 0 to 120 min were determined and the differences between each group receiving insulin and enhancer formulations and the control group, which received insulin alone, were assessed using Student's t test. As recommended by Elashoff (6), the Bonferroni method was used to reduce the significance level for rejection of the null hypothesis and thus reduce the possibility of a significant result arising by chance after multiple t tests.

At the end of the absorption experiments, after 2–4 hr, the rats were sacrificed by an overdose of pentobarbitone sodium and the vaginal tissues were removed and placed in fixative for histology. In addition, a control group of rats were prepared. They were treated in a similar manner to the experimental group but did not receive a vaginal instillate.

Histological Study

The tissues were fixed in Bouin Hollande fluid and processed by conventional steps for histological examination. The thickness of the vaginal epithelium in the control group of rats was quantitatively assessed as follows. Five sections from each animal were randomly selected from a group of nine sections collected through the length of each organ. The thickness of the vaginal epithelium was measured by means of an eyepiece graticule at five sites of each section. Hence, 25 measurements were made for each rat and the data expressed as the mean epithelial thickness. However, it was not possible to assess quantitatively the histological effects of the enhancers such as changes in epithelial thickness, since for some enhancers the epithelium was intact but showing intracellular damage.

RESULTS

After vaginal administration of buffer solution alone, blood glucose concentrations were found to rise steadily throughout the experiment, to reach 137% of basal levels after 4 hr. Similar results were obtained after vaginal administration of insulin solution alone, with mean blood glucose concentrations reaching 115% of basal levels after 4 hr (Fig. 1).

Figures 2 and 3 illustrate the effects of each enhancer on blood glucose concentrations after vaginal administration of insulin solution, 8 IU/kg. The extent and time course of the hypoglycemic effects varied after vaginal administration of each formulation. Maximal decreases in blood glucose levels were seen between 40 and 120 min after administration, and in general, concentrations then rose steadily to near-basal levels by the end of the experiment. The hypoglycemic response to insulin and enhancer formulations provided an indirect measure of the extent of insulin absorption. The mean areas under the curves (AUCs) of blood glucose concentrations from 0 to 120 min after each treatment are shown in Table I. With the exception of the formulation containing citric acid, vaginal administration of insulin and enhancer solutions resulted in significantly lower AUCs and, hence, greater hypoglycemia than administration of insulin solution alone. The differences in the degree of hypoglycemia after vaginal administration of insulin with each of the enhancers were negligible.

The vaginal epithelium of the control group had a mean thickness of 41 μm (SE, 1 μm) and consisted of a basal layer of cuboidal cells covered by several layers of flattened squamous cells and an outer layer of cuboidal cells (Fig. 4a). The changes in tissue morphology after treatment with the insulin

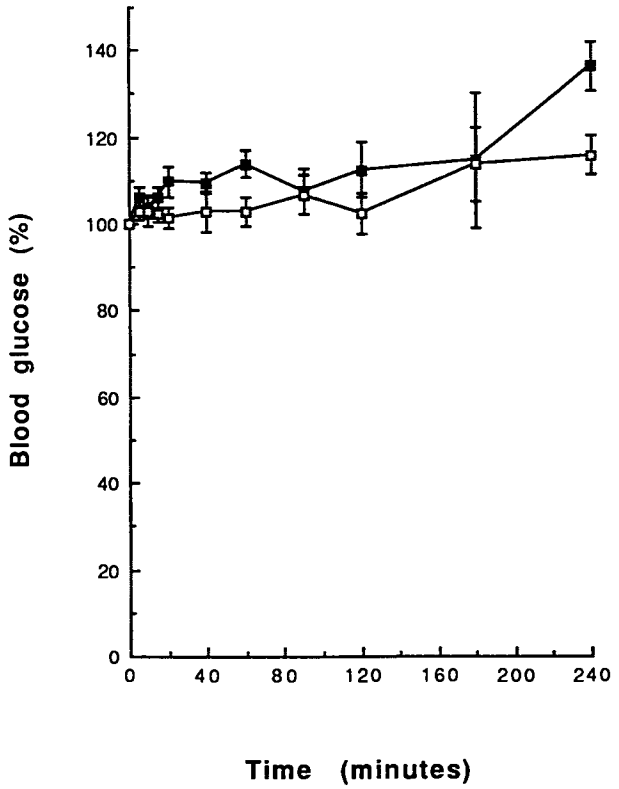

Fig. 1. Basal blood glucose level (%) after vaginal administration of buffer or insulin solution, 8 IU/kg, to rats. (□) Insulin alone, n = 7; (■) buffer alone, n = 3. Each value represents the mean ± SE.

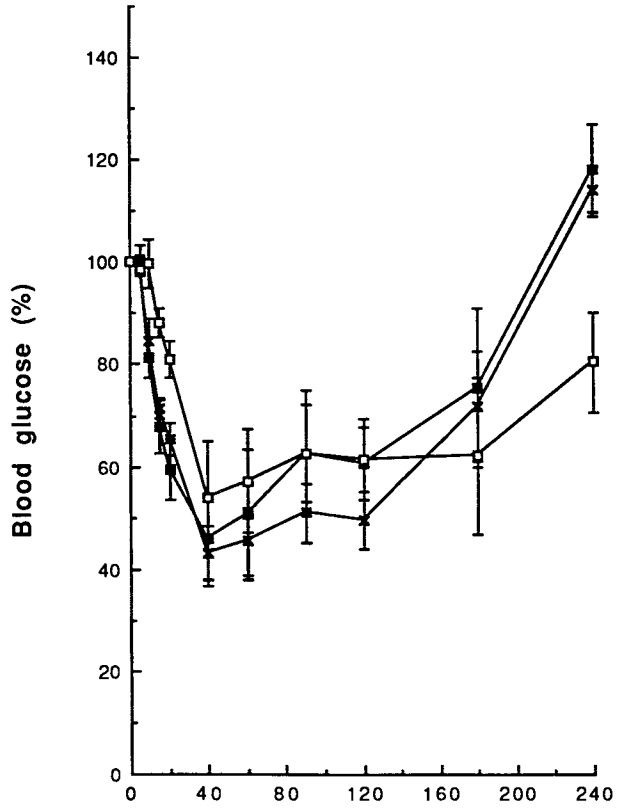

Fig. 2. Change in basal blood glucose level (%) after vaginal administration of insulin (8 IU/kg) with enhancers to rats. (X) Plus 0.5% lysophosphatidylcholine; (□) + 0.5% lysophosphatidylglycerol; (■) + 1% palmitoylcarnitine chloride. Each value represents the mean ± SE of five or six animals.

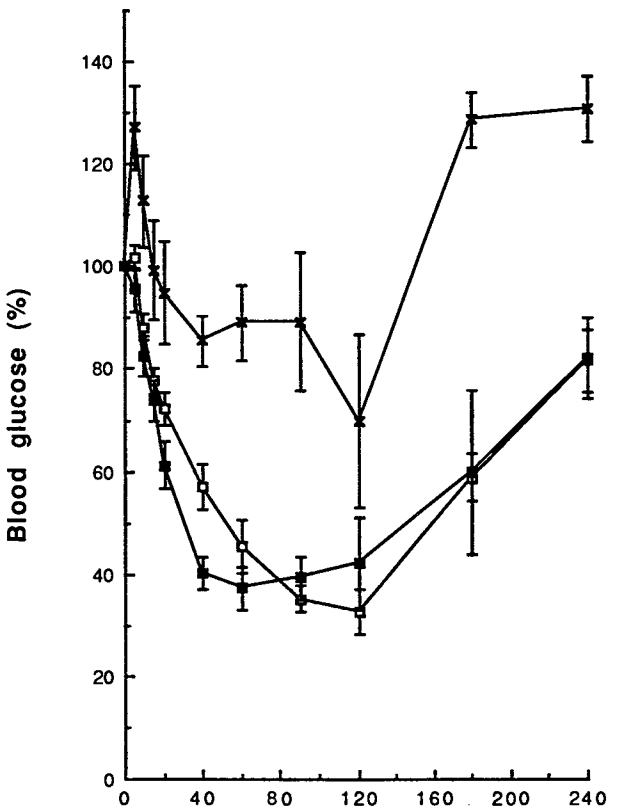

Fig. 3. Change in blood glucose level (%) after vaginal administration of insulin (8 IU/kg) with enhancers to rats. (□) Plus 1% polyoxyethylene-9-lauryl ether; (■) + 1% sodium taurodihydrofusidate; (X) + 10% citric acid. Each value represents the mean ± SE of six or seven animals.

and enhancer formulations are shown in Figs. 4 and 5. The vaginal epithelium was not changed following treatment with the insulin solution alone (Fig. 4b), except for one animal in which there was some loss of the surface layer. The coadministration of insulin with LPG, LPC, PCC, laureth-9, and citric acid resulted in a comparable spectrum of histological changes, whereas STDHF had a distinct, individual effect on the vaginal epithelium. After treatment with the former enhancers, the surface cuboidal layer was often disrupted or lost; the squamous cells were altered with dense nuclei and eosinophilic cytoplasms, and in some cases, the basal cell layers were also affected. In the few areas displaying severe damage, there was a complete loss of cellular structure, with a hyaline-like residue remaining. Insulin and LPG or LPC solutions tended to disrupt the surface cuboidal layer only (Figs. 4c and d), although the latter formulation caused damage in deeper epithelial layers in two animals. Epithelial changes were more pronounced after coadministration of the drug with citric acid, PCC, and laureth-9 (Figs. 5a and b) without evidence of the type of cellular damage seen above.

In many tissue samples, an epithelium with an unchanged morphology was present lining the folds of the vagina, which indicated an uneven distribution of the solution and lack of penetration into these protected areas. This result demonstrated that the volume of the instillate administered did not excessively distend the normal configuration of the vagina.

Table I. AUC of Blood Glucose Levels from t_0–120 min, After Vaginal Administration of Insulin Solution Alone and with Enhancers to Rats (Mean ± SE)

Treatment	AUC × 10^3 (% · min)	n
Buffer solution	13.15 ± 0.41	3
Insulin solution, 8 IU/kg	12.41 ± 0.40	7
+10% citric acid	11.51 ± 0.97	6
+0.5% lysophosphatidylglycerol	8.00 ± 0.88*	5
+1% palmitoylcarnitine chloride	7.21 ± 0.86*	6
+0.5% lysophosphatidylcholine	6.58 ± 0.44*	6
+1% polyoxyethylene-9-lauryl ether	6.32 ± 0.33*	7
+1% sodium taurodihydrofusidate	5.65 ± 0.46*	6

* Significantly different from group receiving insulin solution alone.

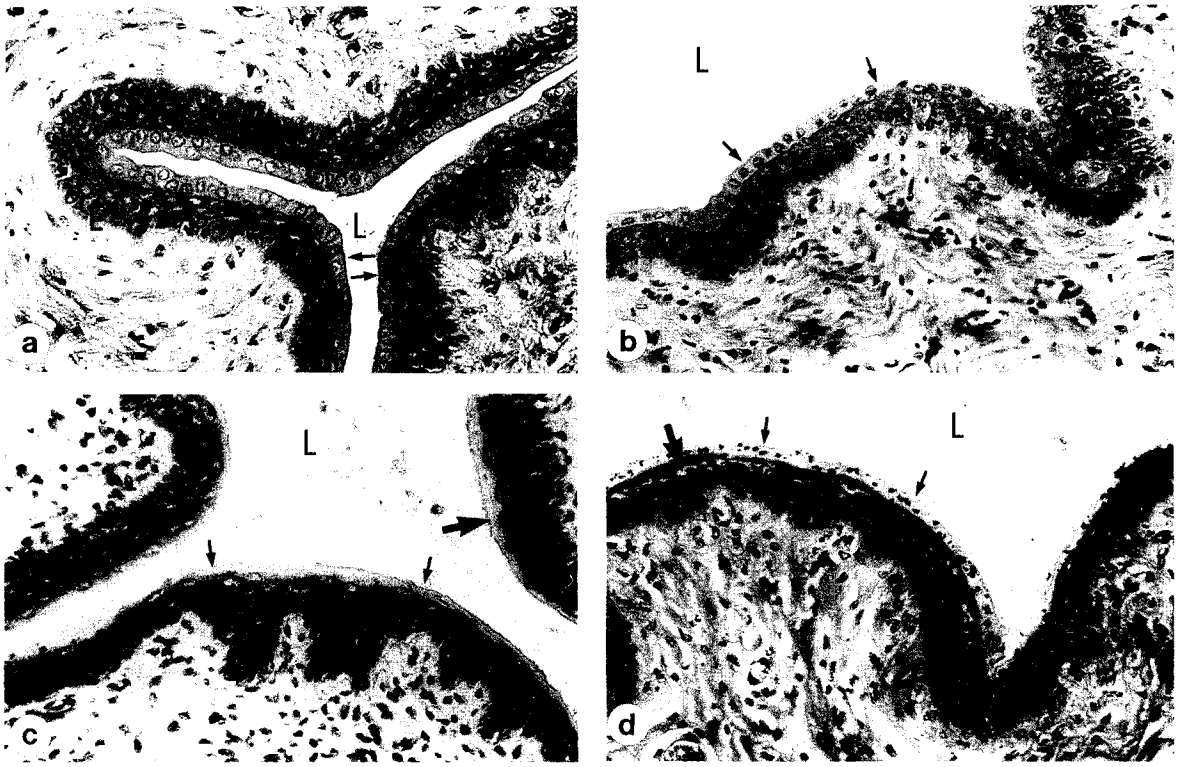

Fig. 4. (a) Control tissue. The vaginal epithelium (E) comprises five or six layers of cells. The surface layer (arrows) lining the vaginal lumen (L) is composed of pale staining cuboidal cells. The deeper layers are more densely stained and organized in the form typical of a stratified squamous epithelium, with a basal layer of cuboidal cells and intermediate layers in which the cell shape changes gradually to a squamous form. ×450. (b) Insulin-treated tissue. The epithelium (E) closely resembles that observed in the control animals, and in particular, the surface layer of cuboidal cells (arrows) lining the lumen (L) is intact and the cell form unchanged. ×445. (c) Insulin/LPG-treated tissue. The surface layer (arrow) has lost its cellular organization and appears as a hyaline layer bordering the lumen (L). At isolated sites (large arrow) some of the squamous cells immediately beneath the surface layer show similar changes but the deeper layers (E) are unaffected. ×450. (d) Insulin/LPC-treated tissue. The surface layer (small arrow) is intact but the cells are vacuolated and the nuclei pyknotic. Similar changes are also apparent at isolated sites (large arrow) in the underlying epithelial layers (E). ×435.

DISCUSSION

The absorption enhancers STDHF, laureth-9, LPC, PCC, and LPG all markedly increased the hypoglycemic effects of vaginally administered insulin solutions while citric acid proved to be less effective. The histology of the vaginal epithelium varied after each treatment, with few changes seen after the administration of insulin alone or with LPG but more severe damage occurring after exposure to the other formulations.

Thus, the most effective enhancers studied were amphipathic compounds with surface-active properties. A number of mechanisms for absorption enhancement have been suggested for these agents, recently reviewed by O'Hagan and Illum (7). It is thought that surfactants may reduce the viscosity of the mucus layer, inhibit peptide degradation by mucosal enzymes, open epithelial tight junctions, or disrupt the lipid bilayer of cell membranes, thus increasing the fluidity of the membrane. The latter mechanism of action has been used to explain the absorption enhancing activity of laureth-9, which was shown to facilitate the leaching of membrane proteins (8) and induce epithelial cell loss after nasal application (5,9). The morphological changes seen after vaginal administration of insulin with laureth-9 were less severe than expected from previous studies in ovariectomized non-estradiol-treated rats, where approximately 50% of areas assessed were bare of epithelium (3). However, in this ovariectomized rat model, the thickness of the vaginal epithelium was reduced considerably to approximately 17 μm (unpublished measurements) and thus was likely to be highly sensitive to drug-induced damage. In the present rat model, the thicker vaginal epithelium appears to be more resistant to enhancer-induced damage. After treatment with laureth-9, the epithelium remained largely intact, although the cellular changes may signal an altered permeability and reduced barrier to insulin absorption. The vaginal epithelium of the ovariectomized estradiol-treated rat can be considered to be more similar in histology to that of the human than can the atrophic epithelium of the nontreated ovariectomized rat (10). The extent of vaginal absorption through the thickened rat vaginal epithelium in the present animal model is likely to be more representative of the potential absorption in women. In addition, the vaginal epithelium in this model has a more constant morphology than the normal untreated rat

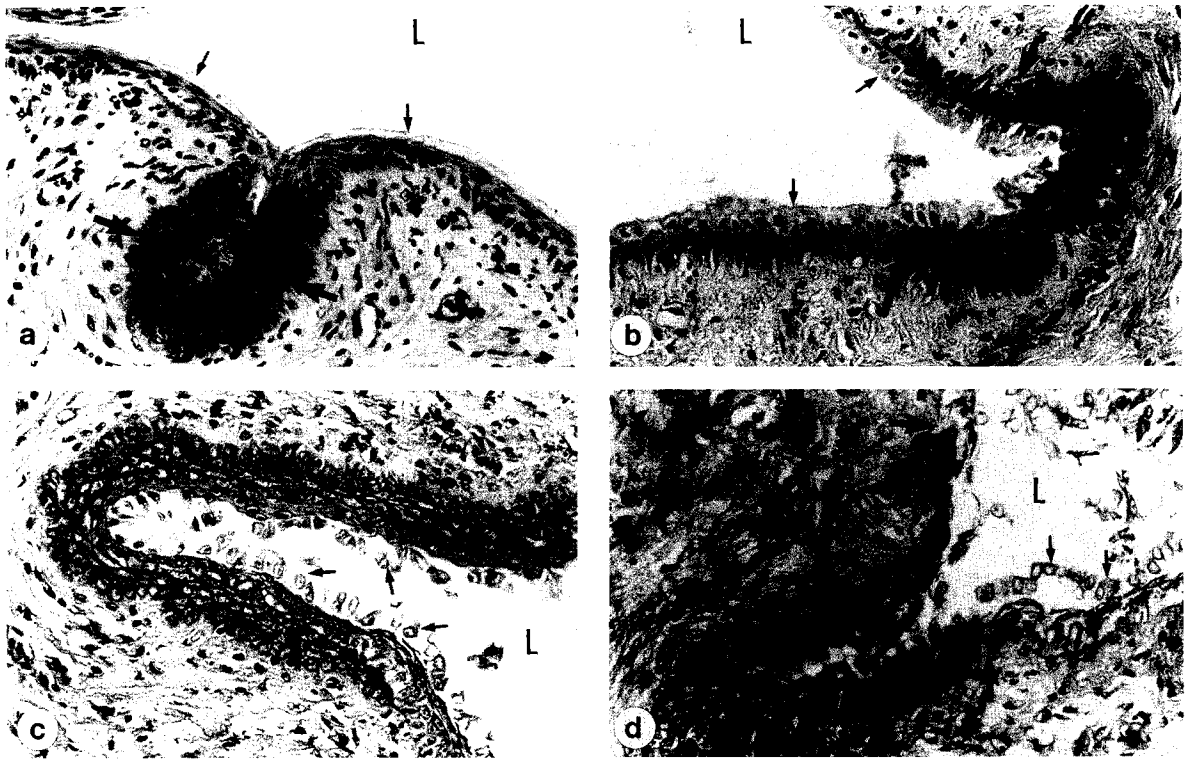

Fig. 5. (a) Insulin/PCC-treated tissue. In the depths of a mucosal fold (large arrows) the epithelium appears unchanged, but elsewhere (small arrows) the surface layer has lost its cellular organization and the deeper layers have pale-staining cells with densely staining, condensed nuclei. L, lumen. ×455. (b) Insulin/laureth-9-treated tissue. The cells of the surface layer (small arrows) exhibit evidence of pyknosis and, in places, cellular desquamation. The large arrows indicate sites of junction between epithelium (E) in a shallow fold, which appears unchanged, and epithelium showing layers of pale-staining cells with densely stained, condensed nuclei. L, lumen. ×450. (c) Insulin/STDHF-treated tissue. This treatment is characterized by epithelial desquamation. Cells (arrows) from the surface layer and layers immediately beneath it are shed into the vaginal lumen (L). The cytoplasmic staining of the deeper epithelial layers (E) is reduced but the nuclear staining appears unchanged. ×430. (d) Insulin/STDHF-treated tissue. The large arrows indicate the junctions between epithelium in the depths of a fold (E), which shows evidence of cellular changes in the surface layer alone, and regions in which desquamation has extended through the whole thickness of the epithelium, with associated cellular debris (small arrows) in the lumen (L). ×460.

and allows the detection of more subtle differences in the histological effects of drugs and enhancers than does the nontreated ovariectomized rat.

STDHF is structurally similar to the bile salts and is thought to increase peptide absorption by similar mechanisms. It has been suggested that insulin may be solubilized by STDHF to form reverse micelles and hence pores within the cell membrane, thereby allowing diffusion of insulin through the cells (11–14). Alternatively, STDHF may act on intercellular tight junctions, allowing paracellular absorption (13). The results of the present study may provide evidence for the latter hypothesis since 2–4 hr after administration of insulin and STDHF, considerable cellular separation and cell loss were observed. However, the histology of the vaginal epithelium at the time of maximal absorption is unknown, and it is not possible to relate directly the histological effects of STDHF with the enhancement of insulin absorption. In comparison, the histology of the rat nasal mucosa has been studied by Lee *et al.* after treatment with STDHF and laureth-9 for 5 min (5). While typical changes after treatment with laureth-9 were cellular erosion, cell–cell separation, dense mucous coating, and loss of cilia, administration of STDHF resulted only in a slight increase in surface mucus. In further studies by Ennis *et al.* (15) the histological effects of various enhancers, including laureth-9 and STDHF, were quantitatively assessed after nasal administration to rats. Gross alterations were reported after exposure to 1% laureth-9 for 5 min, with partial or complete removal of the nasal epithelium observed. The histological effects of 0.5 and 1% STDHF on the rat nasal mucosa were less severe. However, quantitative assessment of changes in mucosal surface integrity, cilia morphology, and the presence of mucus, extracellular debris, and red blood cells after treatment with STDHF demonstrated consistently higher scores and thus greater damage than for the control group. In addition, 1% STDHF was found to induce greater damage than 0.5% STDHF, and with the former, treatment for 5, 10, or 15 min resulted in similar histological changes.

The histological effects of 0.5, 1, and 4% STDHF has also been investigated after rectal application in rats (16). The morphological changes observed after treatment with 0.5 and 1% STDHF were described as relatively moderate and included hyperemia, edema, and epithelial cell detachment. In contrast, 4% STDHF was reported to exert stron-

ger and more persistent effects. Hence, the changes in the vaginal epithelium seen in this study after exposure to STDHF are similar to what has been found by other groups for the epithelium after nasal and rectal administration.

The remaining three surfactants, LPC, PCC, and LPG, had varied effects on the vaginal epithelium while demonstrating marked enhancement of insulin absorption. PCC was found to exhibit the greatest local toxicity, with reductions in epithelial thickness and areas of dead unstained cells often observed. These findings were surprising in view of the results from the studies in non-hormone-treated ovariectomized rats, where PCC caused moderate epithelial damage and treatment with LPC resulted in more substantial cell loss (3). In addition, PCC has been utilized in the gastrointestinal tract of rats and dogs and was found to be an effective enhancer without affecting mucosal integrity (17). However, LeCluyse *et al.* (18) later reported a relationship between the absorption enhancing activity and the membrane perturbing effects of a series of acylcarnitines, including PCC. They suggested that in order to promote absorption, these compounds must be capable of partitioning into the intestinal membrane and causing sufficient disruption of its lipid order.

LPC and LPG are both lysophospholipids which are surface-active at low concentrations. LPC has been shown to be an effective enhancer of both vaginal absorption (3) and nasal absorption (19) of drugs in rats. The mechanism of absorption enhancement by LPC has not been defined but may be similar to that suggested for surfactants. In the present study, LPG emerged as a promising absorption enhancer with minimal effects on mucosal histology. LPC appeared to have more severe effects on vaginal mucosal histology than LPG but the differences in effect between the two enhancers are difficult to explain with the data presently available.

The chelating agent, citric acid, is thought to enhance drug absorption by the removal of calcium ions from intercellular tight junctions, thus allowing the formation of a channel through which drugs may diffuse. Citric acid has been successfully used to increase the vaginal absorption of a LH-RH analogue, leuprolide, and insulin in rats (4,19). Indeed, in these studies citric acid was found to be a more effective enhancer than surfactants such as laureth-9. In contrast, in our studies the vaginal administration of surfactants to both vaginal rat models resulted in considerable enhancement of drug absorption, while citric acid was shown to be less effective despite causing epithelial damage.

Although the efficacy of enhancers in promoting the vaginal absorption of insulin is encouraging, their mechanisms of action and histological effects must be studied further before their chronic use could be considered.

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