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Nasal absorption in rats. II. Effect of enhancers on insulin absorption and nasal histology

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Summary

Intranasal formulations have been investigated using a combined absorption and histological model to determine the effects of different absorption enhancers on insulin uptake and nasal epithelium integrity. Based on changes in blood glucose levels, enhancers were ranked according to their insulin absorption promoting effect, while qualitative histological assessment enabled ranking according to the severity of epithelium disruption. Despite a close relationship between the two effects, removal of the epithelial barrier was not considered to be the only mechanism of action for all of the enhancers studied and other factors require further investigation. The model used enables early assessment of compounds from both an enhancing efficiency and toxicological standpoint.

Introduction

Developments in biotechnology and therapeutics have resulted in the increased use of many peptide and protein drugs. Parenteral routes of administration (i.v., i.m. or s.c.) are generally required for systemic activity because of their poor oral bioavailability. However, associated complications may result in low patient acceptance of these routes and non-compliance is a particular risk when self-administration is needed for chronic therapy. Consequently, alternatives such as the nasal, buccal, ocular and vaginal routes have been considered for systemic peptide and protein drug delivery (Davis et al., 1986; Banga and Chien, 1988).

The nasal route has been extensively investigated (Chien et al., 1989; Pontiroli et al., 1989). Access is straightforward and well tolerated by patients. Additionally, the nasal mucosa is characterised by features important for systemic drug delivery, notably a highly vascular mucosal bed and avoidance of the first-pass elimination effect. However, systemic levels of protein and peptide drugs tend to remain low following nasal administration. A combination of high molecular weight and polar character is thought to restrict passage

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across the nasal epithelium (Fisher et al., 1987; McMartin et al., 1987; Donovan et al., 1990a) while a significant degree of enzymatic degradation and rapid transport away from the site of absorption by the mucociliary clearance system may also contribute to the low levels of drug reaching the circulation (Stratford and Lee, 1986; Illum et al., 1987; Lee et al., 1987).

The usual method adopted to increase the nasal bioavailability of drugs such as insulin, human growth hormone and salmon calcitonin has been the co-administration of a variety of agents commonly termed absorption enhancers (Hanson et al., 1986; Deurloo et al., 1989; Illum et al., 1989; Baldwin et al., 1990). However, such formulations have been shown to result in surface changes in the nasal cavity including membrane protein removal, cell loss, excessive mucus discharge and ciliotoxicity (Hirai et al., 1981; Duchateau et al., 1986; Hersey and Jackson, 1987; Daugherty et al., 1988; Gizurarson et al., 1990; Hermens et al., 1990). Consequently, many compounds are excluded from nasal formulations when chronic therapy is indicated (Lee and Yamamoto, 1990).

Chandler, Illum and Thomas (1991) have described a novel method for assessing the effects of intranasal drug formulations on rat nasal epithelium by light microscopy. This method was used in the present study in combination with blood glucose determinations to assess the intranasal absorption of insulin ($M_w = 5700$) in the presence of a variety of enhancers. The following enhancers were selected for investigation:

Sodium taurodihydrofusidate (STDHF) is a bile salt derivative which is reported to enhance nasal insulin absorption with few effects on the nasal epithelium (Lee et al., 1988).

L-α-Lysophosphatidylcholine (LPC) is a surface-active, amphiphillic compound produced in biological membranes. It has been shown to increase the permeability of rat ileum to macromolecules (Tagesson et al., 1985) and has more recently been investigated as an enhancer for the intranasal absorption of insulin in rats (Illum et al., 1989) and of human growth hormone in rats, rabbits and sheep (O'Hagan et al., 1990; Illum et al., 1990) although its histopathological effects on the nasal tissue were not reported. It is active at low concentrations and is converted in biological membranes to metabolites which are naturally occurring cell constituents (Stafford and Dennis, 1988). However, LPC has been shown to damage gastric mucosa (Bolin et al., 1986) and vaginal epithelium in ovariectomised rats where epithelium thickness is reduced (Richardson et al., 1989).

DEAE-Dextran (diethylaminoethyl-dextran) is a polycationic polysaccharide which forms complexes with insulin by simple mixing of component solutions (Manosroi and Bauer, 1990). The uptake of DEAE-Dextran into mammalian cells has been shown to occur at an increased rate compared to serum albumin; in combination, the polysaccharide stimulated uptake of the protein (Ryser, 1968).

Laureth-9 (polyoxyethylene-9-lauryl ether, L-9), a non-ionic surfactant, has been widely used as an enhancer of intranasal drug absorption and its damaging effects on the nasal epithelium have been reported (Daugherty et al., 1988; Donovan et al., 1990b; Chandler et al., 1991). Therefore, in the present study, corresponding absorption data were obtained without further histological investigation.

Materials and Methods

Materials

Semi-synthetic human insulin (sodium salt, SHI, BN P371) was obtained from Novo-Nordisk, Gentofte, Denmark; the water content was determined as 14% w/w.

Polyoxyethylene-9-lauryl ether (laureth-9), DEAE-Dextran and L-alpha-lysophosphatidylcholine (stearoyl/palmitoyl mixture) were purchased from Sigma Chemical Company Ltd., Dorset, U.K. Sodium taurodihydrofusidate was obtained from California Biotechnology, California, U.S.A. Standard histological preparations and all other materials used were of reagent grade.

Dose preparation

All solution concentrations were calculated for administration of the required dose in a 20 μl

volume to 250 g rats. Insulin solution, 100 IU/ml (8 IU/kg) in phosphate buffer (pH 7.3), was freshly prepared each day; absorption enhancers were then added at the required concentrations. The final dosing formulations were all solutions except the DEAE-dextran enhanced system which formed a fine suspension due to complex formation between the enhancer and insulin molecules.

The selection of enhancer concentrations was based on published reports and previous work within the department.

The following preparations were administered: (1) Insulin solution (100 IU/ml) without the addition of any enhancer. (2) Insulin solution (100 IU/ml) with 1% w/v laureth-9 (0.8 mg/kg). (3) Insulin solution (100 IU/ml) with 0.625% w/v LPC (0.5 mg/kg). (4) Insulin solution (100 IU/ml) with 1% w/v STDHF (0.8 mg/kg). (5) Insulin solution (100 IU/ml) with 5% w/v DEAE-Dextran.

Animal preparation and dosing

Groups of five or six animals were used for each formulation. Animal preparation and nasal dosing was carried out according to the methods described by Illum et al. (1989) for the in vivo assessment of nasal drug absorption. Male Wistar rats (JABU, Sutton Bonington, U.K.) weighing approximately 250 g, were fasted overnight prior to each experiment and anaesthetised intraperitoneally with 60 mg/kg sodium pentobarbitone (Sagatal, 60 mg/ml, May and Baker, Essex, U.K.). A tracheotomy diverted airflow from the nasal passages and aided breathing. The oesophagus was closed by ligation onto the tracheal cannula. The left carotid artery and right external jugular vein were cannulated for blood sampling and fluid replacement (with normal saline), respectively.

The 20 μl dose was delivered to the right nostril only using a Hamilton syringe with attached length of polyethylene tubing, inserting the dosing tube about 0.5 cm into the nostril.

Blood sampling

140 μl blood samples were collected in fluoride oxalate blood tubes (Sterilin Ltd., Middlesex, U.K.) and stored on crushed ice until analysis. Glucose determinations were carried out within 4 h of sampling using a Yellow Springs Instrument 23AM glucose analyser. Samples were taken 10, 6 and 2 min before nasal dose administration to establish baseline glucose levels and then 5, 10, 15, 20, 25, 30, 40, 50 and 60 min after dosing.

Tissue sampling and processing

After collection of the final blood sample, tissue was fixed by cardiac perfusion of Bouin Hollandes fixative solution and processed as described by Chandler et al. (1991) to yield complete cross-sections of the nasal cavity.

Analysis of data

The mean blood glucose concentration from the three pre-dose samples was taken as the baseline level. Using these values the percentage of glucose remaining at each time point after dosing was calculated and plotted against time. The area under this curve (AUC) at 60 min was then determined for each animal. Increased insulin absorption was indicated by an increased fall in blood glucose levels and thus, a smaller AUC value.

Statistical analysis was carried out on the two summary measures of AUC and % reduction of blood glucose concentration at the end of the experiment (Elashoff, 1981; Matthews et al., 1990), using the Unistats-II software package (version 2.01) for Acorn BBC series microcomputers.

Single-factor analysis of variance on all data, including controls, determined any difference between group means. To identify the source of any differences found (eg. between which treatment group and control) multiple comparisons of data were made using the Bonferroni adjustment of Students *t*-test (Elashoff, 1981; Godfrey, 1986). Initially, *F*-tests (analysis of variance) on pairs of group data were carried out to ensure use of the appropriate *t*-test (equal or unequal variance). Calculated two-tailed probability values were then compared to a 5% level of significance ($p = 0.05$) divided by the total number of *t*-tests performed. This results in a reduction of the probability level of significance to account for results which might

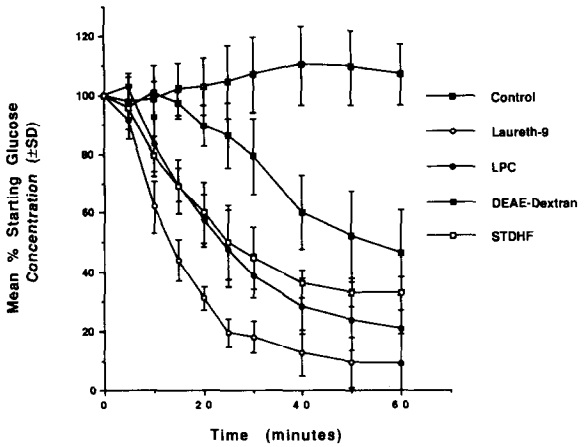


Fig. 1. Blood glucose levels in rats following the administration of 8 IU/kg insulin alone in solution and with different enhancers.

spontaneously occur as more tests are carried out on the data.

For histological analysis cross-sections of the nasal cavity were randomly selected from each animal. The state of the nasal epithelium on the dosed side of the septum was qualitatively compared with the tissue on the undosed side in the same section. Comparison was also made between tissue treated with enhanced formulations and that exposed to insulin in buffer only.

TABLE 1

Mean values for % fall in blood glucose concentration (t_{40}) and AUC of % baseline blood glucose plots (t_0–t_{60}) after the nasal administration of insulin solution alone and with enhancers to rats ($n = 5-6$)

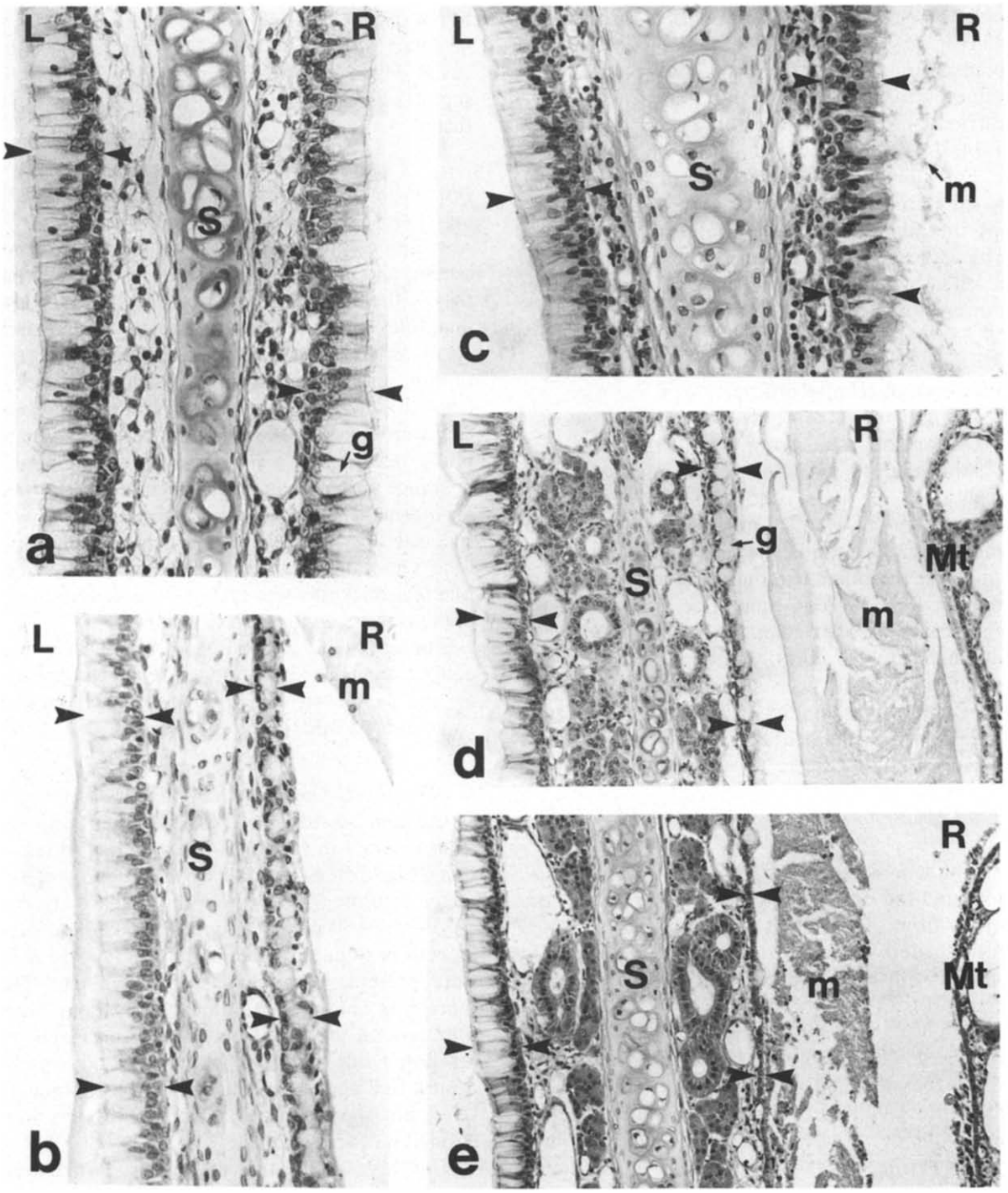
Treatment	% Fall blood glucose (mean ± S.D.)	AUC (% min) (Mean ± S.D.)
Insulin solution, 8 IU/kg	−10.1 ± 13.4	6310.2 ± 564.5
With 1% w/v Laureth-9	87.4 ± 7.9	1892.8 ± 285.1
With 0.625% w/v LPC	71.6 ± 9.2	2914.4 ± 353.6
With 5% w/v DEAE-Dextran	39.8 ± 12.5	4555.4 ± 482.4
With 1% w/v STDHF	63.9 ± 4.6	3211.5 ± 374.4

Results

Insulin absorption

Fig. 1 shows the changes in blood glucose levels following the nasal administration of insulin in combination with different absorption enhancers. The increase in insulin absorption due to the addition of 1% w/v L-9 to the formulation was so dramatic that severe hypoglycaemia resulted in the premature death of a number of animals after 40 min. Heavy reliance on single extrapolated data points for the comparison of % fall in blood glucose levels at the end of the

Fig. 2. Photomicrographs of vertical sections through the anterior rat nasal cavity showing the two sides of the nasal septum (S) 60 min after dosing 20 μl of insulin solution (100 IU/ml in phosphate buffer) into the right side (R), with and without the addition of absorption enhancers. Normal respiratory epithelium, that is, ciliated pseudostratified columnar epithelium densely populated with pale staining goblet cells (g) covers the undosed left side (L) in each case. Treated tissue on the right side can therefore be compared with untreated tissue on the left of the septum; epithelium thickness on the two sides is indicated by arrow heads. (a) No enhancer: few signs of interaction between dose solution and respiratory epithelium are observed. H.E. × 500 (b) LPC (0.625% w/v): disruption of respiratory epithelium on the septum (S) has occurred on the dosed side, with cell loss and mucus discharged into the lumen of the nasal cavity (m). H.E. × 500. (c) 5% w/v DEAE-Dextran; there are few signs of epithelium interaction with only a limited amount of mucus (m) discharged into the lumen of the cavity on the dosed right side (R). The treated epithelium appears slightly more disordered than the untreated tissue but there is little difference in membrane thickness. H.E. × 500. (d) 1% w/v STDHF; treated epithelium is reduced in thickness compared to the untreated tissue and there is evidence of cell loss and mucus discharge (m) into the dosed side of the cavity, though some goblet cells (g) remain intact. The right maxilloturbinate (Mt) is also shown. H.E. × 250. (e) 1% w/v laureth-9 solution (without insulin): respiratory epithelium on the septum of the dosed side has been reduced to a thin layer of cells; discharged mucus (m) containing lost epithelial cells is present in the lumen. On the undosed side (L) the respiratory epithelium remains undisturbed. H.E. × 250.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experiment was considered undesirable, so levels after 40 min only were used. The effect of extrapolation on final AUC values was less marked as glucose levels were so low, hence 60 min data points were calculated (from ln plots) before AUC values were determined and statistical analysis carried out. A summary of results is given in Table 1.

From Fig. 1 and Table 1 it can be seen that blood glucose levels tended to rise slightly following the administration of insulin in buffer only. The addition of each enhancer to the formulation resulted in a significant decrease in blood glucose concentration after 40 min compared to control ($P < 0.005$). Comparisons made between different enhancer systems, however, suggested some difference in relative efficacy.

In Fig. 1 the rate of reduction in blood glucose levels appears to be slower with 5% w/v DEAE-Dextran than for the other enhancers. Statistical analysis of the data confirmed that the differences in % fall after 40 min and AUC after 60 min were significant ($P < 0.005$). 1% w/v Laureth-9 on the other hand appears to result in an increased effect. AUC values for laureth-9 were significantly smaller than for the other systems although after 40 min only, the difference compared to LPC was not statistically significant using Bonferroni's method. 1% w/v STDHF and 0.625% w/v LPC had equivalent, intermediate effects on blood glucose concentration.

Histological effects

Interactions between the different formulations and the nasal mucosa resulted in a range of effects from slight mucus discharge and decrease in epithelium thickness to severe epithelium disruption with extensive cell loss and mucus discharge (Fig. 2).

Insulin 100 IU / ml in phosphate buffer (pH 7.3)

The nasal administration of insulin in phosphate buffer resulted in increased mucus secretion in the dosed side of the cavity. However, the mucus was usually still distributed over the luminal surface of the septal epithelium and not dispersed into the rest of the cavity indicating the low volumes discharged. There was a slight reduction in epithelium thickness when compared to the undosed side (Fig. 2a) but cilia appeared to be intact on both the left and right sides of the cavity. Histological effects were restricted to the septal regions with turbinates apparently unaffected.

Insulin 100 IU / ml with LPC 0.625% w / v (0.5 mg / kg)

Solutions containing LPC induced mucus secretion and epithelium disruption. Cells lost from the epithelium were observed mixed with large quantities of mucus in the lumen of the nasal cavity on the dosed side. Nasal epithelium on the septum and turbinates was reduced in thickness (Fig. 2b) and had undergone rearrangement; nuclei were packed towards the basement membrane resulting in a more typical columnar appearance rather than the usual pseudostratified arrangement. In some places the epithelium was reduced to a thin layer of squamous cells with total secretion of mucus stores, but though epithelium thickness was greatly reduced the membrane was never completely removed.

The undosed "control" side of the cavity was unaffected; only a thin mucus layer was present on the septal surface or in the dorsal meatus comparable to that in undosed animals.

Insulin 100 IU / ml with DEAE-Dextran 5% w / v

Coadministration of DEAE-Dextran with insulin resulted in few signs of epithelium interaction (Fig. 2c). Some mucus was observed, secreted into the dosed side of the nasal cavity, but there were still plenty of intact mucus-filled goblet cells present in the membrane. A few lost cells were present, mixed with secreted mucus, but there was only a slight reduction of epithelium thickness on the nasal septum and turbinates on the dosed side. The epithelium still had a pseudostratified appearance and formed a continuous layer, but the clear cell structure was less well defined on the dosed side.

The histological effects of this formulation were generally restricted to the ventral half of the cavity and lateral nasoturbinate.

Insulin 100 IU / ml with STDHF 1% w / v

Compared to the DEAE-Dextran enhanced formulation, STDHF resulted in more severe disruption of the nasal epithelium after contact for 1 h (Fig. 2d). Large volumes of mucus were apparent in the body of the nasal cavity, together with cell loss, epithelium rearrangement and considerable reduction of epithelium height. Generally the full lengths of the dosed septum and turbinates were affected. Specific staining for acidic mucopolysaccharides with alcian blue showed that some mucus remained in many of the goblet cells while others had totally discharged their mucus content, reducing the epithelium to a thin layer of flattened cells. These effects were similar to those resulting from the use of LPC as enhancer; qualitative comparison of the histological damage however, concluded that the effects of STDHF were slightly less severe than those resulting from contact with LPC.

Some mucus was apparent on the undosed septum or in the dorsal meatus, but with no cell loss. The undosed turbinates were completely unaffected. Epithelium height on this undosed control side was consistently greater than on the dosed, test surface.

Discussion

All of the enhancers investigated were effective in promoting the intranasal absorption of insulin in rats. The extent of reduction of blood glucose levels and degree of interaction with the epithelial barrier however, varied between formulations.

Based on changes in blood glucose levels (AUC_{t0-t60}) the enhancers were ranked according to their insulin absorption promoting effect as follows:

L-9 > LPC = STDHF > DEAE-Dextran

≫ No enhancer

Qualitative assessment of the histological effects of the different enhancers also enabled ranking according to the extent of epithelium damage. The effect of 1% w/v L-9 on nasal tissue was observed in previous experiments without simultaneous absorption studies (Chandler et al., 1991). Insulin was not included in the test formulation but otherwise the system was the same with a 20 μl dose in contact for 60 min. These results were used for comparison with observations from the present study (Fig. 2e).

Thus, the enhancers were ranked according to histological effect as follows:

L-9 ≫ LPC ⩾ STDHF > DEAE-Dextran

> No enhancer

These results would appear to indicate a close relationship between increasing insulin absorption and the degree of epithelial damage. This could suggest a common mechanism of action, namely that the insulin absorption is facilitated by disruption of the epithelial barrier. Modification of the nasal membranes in this way, including protein removal, has been proposed as the mechanism of action of surfactant type enhancers (Hirai et al., 1981).

However, there are several factors to be taken into consideration. Firstly, the time of tissue sampling. The 60 min contact time was chosen to enable blood sampling over a reasonable period in order to determine the effects of enhancers on insulin absorption. Blood glucose levels, however, started to drop 5–15 min after dose administration and fell most rapidly over the first 30 min (Fig. 1), except with DEAE-Dextran when the onset of effect was delayed. In general therefore, the tissue was not fixed at the time of maximum insulin absorption. It is possible that the epithelium damage observed was a secondary effect of some of the enhancers, occurring after insulin absorption had been promoted by some other, less destructive mechanism.

The histological effects of 1% w/v L-9 and 1% w/v STDHF have been investigated by other researchers after shorter contact times with the nasal epithelium. Severe epithelium disruption was observed 5 min after the administration of 1% w/v L-9 (Daugherty et al., 1988; Ennis et al., 1990; Chandler et al., 1991). 1% w/v STDHF resulted in less membrane damage than 1% L-9

after 5 min exposure (assessed by scanning electron microscopy) with reduced surface mucus secretion and extracellular debris (Ennis et al., 1990). The use of 0.5% w/v STDHF further reduced changes in the structural integrity of the nasal mucosa after 5 min, but after 10 or 15 min of contact the histological effects were considered to be the same as for the 1% w/v solution.

In the present study, L-9 was considered to be the most effective enhancer of intranasal insulin absorption, but the use of STDHF also caused blood glucose levels to drop within 5 min. This may suggest a different or additional mechanism of action for STDHF, other than simple epithelium disruption, at least in the initial stages.

Despite a slower onset of activity, the use of DEAE-Dextran resulted in a significant fall in blood glucose levels 60 min after dosing, yet much less epithelium disruption was observed. Blood glucose levels were still showing a downward trend after 60 min suggesting that the maximum effect had not yet been achieved. In this case the effects on the nasal epithelium might be a more accurate reflection of changes occurring during the absorption process. This again suggests a possible alternative mechanism of action not involving epithelium disruption to any great extent. Such a profile with a less rapid fall in glucose concentration may prove to be a more useful system.

The absorption enhancing effects of LPC and STDHF were considered to be equivalent in this study. It should be recognised, however, that the two solution concentrations were not equivalent, with less LPC administered (on a % w/v basis). LPC caused increased epithelium damage though the difference between the two enhancers was considered slight. Different or additional mechanisms of action may again be indicated.

Reduction of the epithelial membrane by cell loss and mucus discharge may not simply increase macromolecular permeation. Increased cell debris in the nasal cavity and at the epithelium surface presents a diffusional barrier through which further drug molecules must pass before reaching the remaining epithelium and underlying blood vessels. Though the viscosity of mucus may be reduced by surfactant enhancers, making diffusion easier (Martin et al., 1978), the large volumes secreted into the nasal cavity may restrict more rapid drug absorption, particularly if any drug-mucus binding interactions occur (Kearney and Marriott, 1987).

Other intracellular constituents which may be discharged following epithelium disruption include mucosal enzymes. Peptide degradation by enzymes is considered to be a significant factor in the low bioavailability of protein and peptide drugs (Stratford and Lee, 1986; Lee and Yamamoto, 1990). Increased contact with such enzymes may reduce the amount of available drug, which may only be partially offset by the enzyme inhibiting activities of some enhancers such as STDHF (Hanson et al., 1986; Lee et al., 1987). Hence, differences in peptidase inhibition could, to some extent, account for the variation in enhancing efficiency observed between the enhancers investigated.

5% w/v DEAE-Dextran was a more viscous system than the other formulations. This could enhance insulin uptake by reducing the rate of drug clearance from the site of absorption (Pennington et al., 1988) but in this animal model the mucociliary clearance mechanism was impaired by anaesthesia and the surgical procedures performed (Fisher et al., 1985). Hence, the enhancing effect of this compound could not be attributed entirely to this property. The increased viscosity could, however, explain the reduced distribution of histological effects which were observed mainly in the ventral half of the dosed side of the cavity and around the lateral nasoturbinate; the dose apparently did not flow easily into the dorsal parts of the cavity from the site of administration. A decreased contact area resulting from such reduced distribution may also have influenced the rate of drug absorption.

The enhancing effect of DEAE-Dextran could be due to electrostatic interaction between the polycationic polysaccharide and negatively charged cell membrane surfaces; this may result in a change of membrane permeability to macromolecules and stimulation of pinocytic uptake (Larsen, 1967; Ryser, 1968). Also, insulin-dextran complex formation was shown by Suzuki et al. (1972) to reduce the enzymatic degradation of the

drug in homogenates of adipose tissue. Although in the study the complex did not pass readily through intact cell membranes, similar protection against enzymes in the nasal cavity could minimise drug losses before other mechanisms of uptake have effect.

The rate of release of free insulin from the complex will be a critical factor in determining the rate of onset of drug activity. This may partially explain the delayed effect observed with the use of DEAE-Dextran in this study, particularly as binding between the polysaccharide and the polypeptide is maximal at pH 7.4. Complex formation thus provides an inherent element of controlled release which could be exploited in future formulations for nasal drug delivery.

Conclusions

The effects of some absorption enhancers on the intranasal absorption of insulin and nasal histology have been demonstrated in a combined model. Some correlation between enhancing efficiency and epithelium disruption was found but removal of the epithelial barrier is not considered to be the only mechanism of action for all enhancers and may just be a side effect of some. While histological effects observed 5 min after dosing are of interest in establishing the mechanisms of absorption enhancement, effects after 1 h are of increased importance from a toxicological point of view. Repeated dosing and recovery studies are necessary to establish the full toxicological implications of regular use of such products. The rat model used for the simultaneous assessment of peptide absorption and histological effects should prove useful for further investigations.

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