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Paracellular and transcellular pathways facilitate insulin permeability in rat gut

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Abstract

The aim of this study was to conduct a systematic investigation of the absorption of insulin in the rat intestine in the presence of permeation enhancers and protease inhibitors. An in-situ perfused rat gut model was used for the co-perfusion of insulin and PEG 4000 in the presence or absence of bile salts, bile salt:fatty acid surfactant systems and protease inhibitors. Perfusion experiments were conducted for 180 min with perfusate and blood collection at regular intervals. Permeability coefficients for insulin were calculated from plasma insulin and PEG 4000 permeability coefficients were calculated from lumenal disappearance data. In the absence of enzyme inhibitors, insulin permeability was consistently lower than PEG 4000, but increased in proportion to PEG 4000 permeability. Large increases in insulin permeability were obtained for mixed micellar systems and protease inhibitors. In the presence of protease inhibitors and simple micelle systems, PEG 4000 permeability was three-fold greater than insulin permeability. In the presence of absorption enhancers, PEG 4000 permeability increased up to a maximum value of 3.63×10^{-6} cm s^{-1}, a value five-fold less than that of the estimated aqueous boundary layer permeability for PEG 4000. This suggests that PEG 4000 permeability is primarily membrane controlled. Insulin permeability is enhanced to a maximum value of 9.17×10^{-6} cm s^{-1}, suggesting that paracellular transport routes do not account exclusively for insulin permeation across the intestinal epithelium. The results add support to suggestions that routes other than the paracellular route may contribute to insulin absorption in rat gut.

Introduction

The oral route is by far the preferred route of drug administration in contemporary therapeutics. Non-oral routes such as the nasal, pulmonary rectal, buccal, vaginal and transdermal routes are considered when the oral drug bioavailability of candidate drug molecules is poor or variable due to impermeability of the intestinal mucosa or extensive first-pass metabolism. In addition to the hostile environment that the gastrointestinal tract poses to peptides and proteins, the development of oral dosage forms of proteins is further complicated by their large size and hydrophilicity. Approaches that have been used to improve the oral delivery of peptide and protein drugs have included the use of site-specific delivery within the gastrointestinal tract, protease inhibitors, penetration enhancers, carrier systems and formulation approaches (Marschütz & Bernkop-Schnürch 2000; Peppas 2004; Ma et al 2005). However, these studies have been directed predominantly at enhancing the bioavailability of insulin rather than elucidating the mechanisms underlying the enhanced absorption of the protein.

Previously we have delineated the relative contributions of a range of formulations to insulin stability vs permeation enhancement in the rat intestine (Lane et al 2005). The competing phenomena of absorption and degradation of insulin in the lumen preclude determination of the intestinal permeability coefficient. In the present study, effective permeability coefficient (P_{app}) values for insulin were estimated from the plasma insulin concentrations.

Bendayan et al (1994) and Ziv & Bendayan (2000) have suggested that routes other than paracellular transport may be involved in insulin absorption. The paracellular absorption of hydrophilic probe molecules is influenced by molecular weight, geometry and flexibility (Hollander et al 1988; Ghandehari et al 1997) and the inclusion of bile salts, as both simple and mixed micellar media, greatly increases the absorption of such probes, consistent with an increase in the paracellular pore size (Lane et al 1996). In this work we report effective

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permeability coefficients for both insulin and the macromolecular probe PEG 4000, a marker for paracellular absorption. Media containing simple and mixed micellar systems and enzyme inhibitors were investigated to explore the parallels between PEG and insulin permeability.

Materials and Methods

Human insulin (28.1 IU mg^{-1}), the trihydroxy bile salts cholate (NaC), glycocholate (NaGC) and taurocholate (NaTC), the fatty acid linoleic acid (LA) and the protease inhibitors soy bean trypsin inhibitor (STI, 10 000 IU mg^{-1}) and bacitracin (BAC, ≥50 IU mg^{-1}) were obtained from Sigma-Aldrich (Poole, UK). Aprotinin (AP) was obtained from Bayer (Trasylol, 10 000 IU mL^{-1}). C^{14}-PEG 4000 was obtained from Amersham UK and purified using a Sephadex-G25 PD-10 column (Pharmacia, Sweden) according to the method of Ghandehari et al (1997). Except where otherwise indicated, all other compounds used in this study were purchased from Sigma-Aldrich (Poole, UK).

Preparation of simple and mixed micellar systems

Simple and mixed micellar systems of bile salts and a fatty acid were prepared as reported previously (Lane et al 2005). Protease inhibitors were perfused with insulin or added to simple and mixed micellar formulations before addition of insulin. The concentrations of protease inhibitors were as follows: STI 1 mg mL^{-1}, BAC 1.4 mg mL^{-1} AP 1 mg mL^{-1}.

In-situ absorption studies

All animal experiments were performed at Trinity College, Dublin in association with the BioResources Unit, which is registered with the Department of Health, the competent authority designated under EU directive 86/609. The facility is under the full-time direction of a veterinary surgeon, who maintains the health and welfare programme. Ethical approval was obtained through the veterinary surgeon.

In-situ absorption studies were conducted according to the rat gut perfusion method described by Komiya et al (1980). Male Wistar albino rats (250–300 g) were used and fasted overnight but allowed free access to water. An intraperitoneal injection (50 mg kg^{-1}) of pentobarbital sodium (60 mg mL^{-1}) was used to anaesthetize the animals. The proximal jejunum was exposed via a midline abdominal incision and cannulated with a glass cannula (i.d. 2 mm, o.d. 4 mm). A 33.3-cm length (l) of intestine was used for all experiments. After gentle washing the distal end of the intestine was cannulated. ^{14}C-PEG 4000 was included in all systems under investigation as a marker of paracellular permeability as previously reported (Lane et al 1996). A flow rate (Q) of 0.2 mL min^{-1} was used. The buffer system used for perfusion experiments was Sørenson's phosphate buffer pH 7.4 adjusted to isotonicity using an osmometer (Model 110, Fiske Associates) where necessary. The concentration of insulin in perfusion solutions was 0.1 mg mL^{-1}. Experiments were conducted for 3 h with perfusate collection at 10-min intervals. Collection sample vials were weighed prior to use and after sample collection for gravimetric correction of fluid flux during perfusion. Blood samples were collected from the jugular vein at 30-min intervals and centrifuged at 3000 *g* for 20 min in a refrigerated centrifuge. Plasma was collected and stored at −20°C prior to analysis for insulin.

Analytical methods

Insulin was assayed using a commercial radioimmunoassay kit (Phadaseph RIA, Pharmacia). The detection range of the assay was 1.5–240 μU insulin mL^{-1}. Samples were diluted where appropriate to fall within the linear range of the assay. The inter- and intra-assay coefficients of variation were both <5%. ^{14}C-PEG 4000 was determined by liquid scintillation counting (Packard Tri-Carb 2500 TR liquid scintillation analyzer). Quench correction was conducted using the method of external standardization.

Calculation of apparent permeability coefficient of PEG 4000

Permeability coefficients for PEG 4000 were calculated from the fraction of solute remaining in the intestinal lumen of length l, and effective lumenal radius r, using equation 1:

$$P_{app} = \frac{-Q}{2\pi rl} \times \ln \frac{(Cl)}{(Co)} \quad (1)$$

where Co is the input perfusate drug concentration, Cl is the outlet perfusate and Q is the flow rate (mL s^{-1}).

Estimation of insulin permeability coefficient (P_{app}) from plasma insulin data

For the two-compartment open model with constant rate intravenous infusion the following is defined (Wagner 1979):

$$C_{eq} = k_0/V_1 \cdot k_{el} \quad (2)$$

where C_{eq} represents an asymptotic or steady state concentration, k_0 is the infusion rate, V_1 is the volume of the central compartment and k_{el} is the elimination rate constant for the central compartment. A bolus intravenous study was carried out to determine the V_1 and k_{el} values for insulin in the rat. Insulin concentrations at steady state, corrected for endogenous insulin levels, were subsequently fitted to the model with zero-order input (k_0) and a mean plasma input rate constant was calculated for each formulation.

The permeability coefficient for insulin was calculated from equation 3, modified from Tsutsumi et al (2003):

$$P_{app} = \frac{k_0}{2\pi rl[C_L]} \quad (3)$$

where $[C_L]$ is the steady-state concentration of insulin exiting the lumen.

Statistical analysis

Each value was expressed as the mean or the mean ± s.e.m. Student's *t*-test was used to test the significance of the difference between two means. Analysis of variance was used when more than two means were compared. *P* values less than 0.05 were considered to be statistically significant.

Results and Discussion

The steady-state concentrations of insulin exiting the lumen (C_L) as determined from Cl values are illustrated for all formulations in Table 1. The corresponding steady-state plasma insulin concentrations for all perfusions (C_{eq}) are illustrated in Table 1 and have been reported previously for the simple and mixed bile salt media (Lane et al 2005). From the bolus intravenous study, k_{el} was calculated to be $0.24 \pm 0.08\ min^{-1}$ and the volume of distribution to be $173.92 \pm 26.88\ mL\ kg^{-1}$. Schilling & Mitra (1992) have also reported that plasma insulin data in the rat was best described by two-compartment model pharmacokinetics after bolus intravenous injection. The permeability parameter estimates calculated for both insulin and PEG 4000 allow a number of observations to be made (Table 1).

The permeability of PEG 4000 was not significantly enhanced by NaGC micellar systems ($P > 0.05$). The NaGC systems enhanced the permeability coefficient of insulin, which is in keeping with the reported enzyme inhibition properties of NaGC (Morimoto et al 2000; Lane et al 2005).

All mixed micellar systems comprising 40 mM linoleic acid:40 mM bile salt significantly enhanced PEG 4000 permeability and insulin permeability ($P < 0.05$). This is in accordance with previous findings where researchers have reported significant enhancement effects of mixed bile salt/fatty acid systems on insulin, calcitonin and heparin (Muranishi 1990; Hastewell et al 1994; Scott Moncrieff et al 1994). The total amount of insulin lost from the perfusion solutions at steady state is composed of fractions degraded and absorbed. As noted previously, the rate of absorption of TC or TC:LA from perfusion systems might be a contributing factor to the lower C_L values of this formulation relative to the GC:LA formulation (Lane et al 2005). As also reported previously, mixed micellar systems promoted insulin instability to a greater extent than simple micellar systems (Lane et al 2005). The protease inhibitors promoted insulin stability with no insulin degradation evident when incubated with any of the inhibitors, which is reflected in the higher C_L values for these systems relative to the control insulin perfusion experiments. Simple micellar systems and protease inhibitors did not affect insulin stability while composite formulations of mixed micellar systems and protease inhibitors appeared to promote insulin instability (Table 1).

Protease inhibitors did not affect the permeability characteristics of PEG 4000; however, AP and BAC significantly enhanced insulin absorption ($P < 0.05$) with BAC having the most pronounced effects on insulin permeability. The permeability coefficient for insulin reflects the appearance of insulin in plasma rather than the disappearance of insulin from perfusate, as for PEG 4000. Formulations consisting of protease inhibitors combined with simple or mixed micelles significantly enhanced both PEG 4000 and insulin permeability ($P < 0.05$).

The physical model for the passive absorption of a drug through the intestinal wall involves an aqueous boundary layer in series with the biomembrane (Ho et al 1977). The biomembrane consists of parallel lipoidal and aqueous pore pathways followed by a blood sink. The observed permeability coefficient P_{app} may be expressed in terms of transport barriers in series:

$$P_{app} = 1/(1/P_{ABL} + 1/P_m) = P_{ABL}/(1 + P_{ABL}/P_m) \quad (4)$$

Table 1 Mean concentration of insulin exiting the lumen (C_L), steady-state plasma insulin concentrations for all perfusions (C_{eq}), and insulin and PEG 4000 permeability

Formulation	C_L (U mL^{-1})	C_{eq} (mU mL^{-1})	Insulin	PEG 4000
			$P_{app} \times 10^6$ (cm s^{-1})	$P_{app} \times 10^6$ (cm s^{-1})
Insulin	1.88 ± 0.20	0.05 ± 0.02	0.12 ± 0.08	0.71 ± 0.15
Insulin/NaGC	1.97 ± 0.11	1.36 ± 0.24**	0.31 ± 0.03†	1.10 ± 0.16
Insulin/NaC:LA	1.01 ± 0.25*	4.93 ± 0.50**	1.24 ± 0.11†	3.42 ± 0.45$^+$
Insulin/NaGC:LA	0.51 ± 0.06*	9.78 ± 0.57**	1.76 ± 0.31†	3.35 ± 1.20$^+$
Insulin/NaTC:LA	0.28 ± 0.04*	9.94 ± 0.63**	0.92 ± 0.90†	3.24 ± 0.83$^+$
Insulin/AP	2.28 ± 0.31	1.82 ± 0.37**	0.36 ± 0.06†	0.67 ± 0.45
Insulin/STI	2.22 ± 0.39	1.05 ± 0.28**	0.20 ± 0.05	0.63 ± 1.20
Insulin/BAC	2.33 ± 0.51	2.14 ± 0.35**	0.43 ± 0.11†	0.81 ± 0.26
Insulin/NaC/AP	2.00 ± 0.59	2.62 ± 0.42**	0.62 ± 0.16†	1.78 ± 0.31$^+$
Insulin/NaC/BAC	1.94 ± 0.42	3.06 ± 0.45**	0.75 ± 0.24†	2.25 ± 0.43$^+$
Insulin/NaC/LA/AP	1.18 ± 0.20*	8.66 ± 1.05**	2.15 ± 0.82†	3.14 ± 0.37$^+$
Insulin/NaC/LA/Bac	1.04 ± 0.28*	18.74 ± 1.91**	4.73 ± 0.77†	3.63 ± 0.11$^+$

*Significantly different from insulin perfusate concentration; **significantly different from control insulin plasma concentrations; †significantly different from P_{app} for insulin when perfused with PEG 4000; $^+$significantly different from P_{app} for PEG 4000 when perfused with insulin; $P < 0.05$. Data expressed as mean ± s.e.m.

where P_{ABL} and P_m are the permeability coefficients of the aqueous boundary layer and mucosal epithelium, respectively. P_m is further described by the permeability coefficients of the transcellular, P_{cell}, and paracellular, P_{para}, routes, respectively:

$$P_m = P_{cell} + P_{para} \quad (5)$$

When $P_m >> P_{ABL}$ then:

$$P_{app} \cong P_{ABL}$$

The extent to which absorption is ABL controlled can be estimated by P_{app}/P_{ABL}. The complementary extent to which absorption is controlled by the mucosal epithelium is found by using $(1 - P_{app}/P_{ABL})$ or P_{app}/P_m.

The molecular size of the diffusing drug influences its permeability coefficient in the aqueous boundary layer, the pores through the aqueous diffusion coefficient D and the Renkin filtration factor, F, respectively (equations 6 and 7):

$$P_{ABL} = D/h \quad (6)$$

$$P_p = (1-\alpha)RD.F/L \quad (7)$$

where h is the thickness of the aqueous boundary layer, P_p is the aqueous pore permeability, α is the volume fraction of the lipoidal phase in the membrane, R is the ratio of effective area for absorption to geometrical surface area of the intestinal lumen and L is the effective thickness of the biomembrane (Ho et al 1977).

The diffusion coefficient and hydrodynamic radius of PEG 4000 have previously been reported as $2.10\times10^{-6}\,cm^2\,s^{-1}$ and 15.9 Å, respectively (Ghandehari et al 1997). The equivalent spherical radius of the insulin monomer has previously been reported as 15.8 Å (am Ende & Peppas 1997). Apparent permeability coefficients for PEG 4000 vs permeability coefficients estimated for insulin in different systems are plotted in Figure 1A. The solid line denotes the relationship that would exist for a one-to-one correlation between PEG 4000 and insulin permeability in the different formulations. Such a correlation might be expected if insulin permeability were enhanced to the same extent as PEG 4000 permeability by each formulation, as suggested by the similar hydrodynamic radii of the molecules. Deviations from this relationship should therefore serve to highlight where formulation components differentially promote PEG 4000 permeability vs insulin permeability. As a corollary, differences in observed permeability enhancements are likely to be the result of different routes of absorption being accessible to the two different compounds. From Figure 1A it is evident that in the absence of enzyme inhibitors the insulin permeability is consistently lower than that of PEG 4000 but also increases in proportion to the increase in PEG permeability. Large increases in insulin permeability were obtained for composite formulations of mixed micelles and protease inhibitors relative to the enhancement of PEG 4000 permeability. When the permeability data for composite formulations of bile salts and protease inhibitors are plotted (Figure 1A, broken line), the permeability values for PEG 4000 are observed to be threefold greater than insulin permeability values.

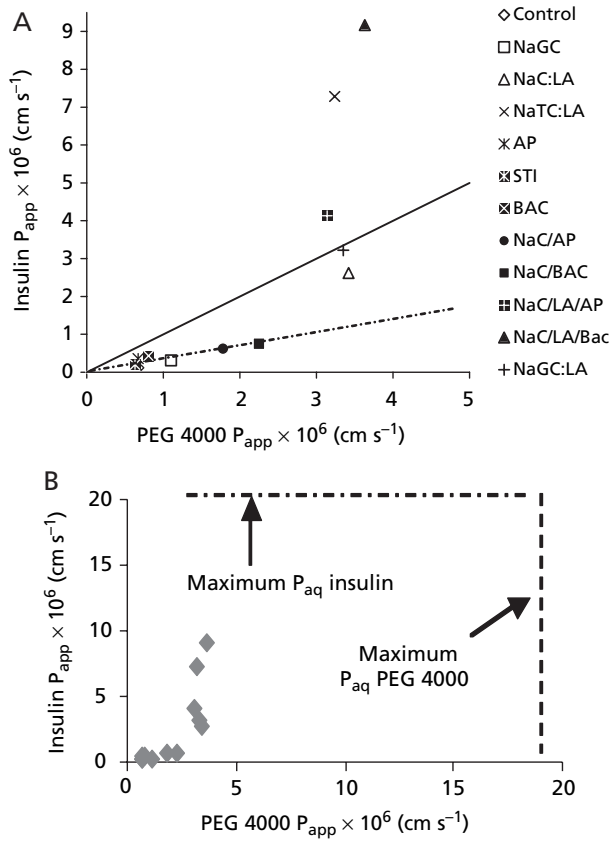

Figure 1 (A) PEG 4000 permeability coefficients vs estimated insulin permeability coefficients. Solid line depicts the theoretical relationship for a 1:1 correlation of permeability coefficients; dashed line represents the linear fit of protease inhibitors and simple micellar systems. (B) Permeability coefficients of PEG 4000 vs insulin with upper limiting aqueous boundary transport (P_{aq}) for PEG 4000 (vertical line) and for insulin (horizontal line).

The maximum value for the P_{ABL} of PEG 4000 may be estimated from equation 4 using the value of 1090 μm for h previously determined for the thickness of the aqueous boundary layer at the flow rate used in the present study (Tsutsumi et al 2003). This generates a value of $1.92\times10^{-5}\,cm\,s^{-1}$ as the upper limiting value of P_{ABL} for PEG 4000 (Figure 1B). The P_{ABL} for insulin is estimated to be $1.91\times10^{-5}\,cm\,s^{-1}$ (Figure 1B).

In the presence of the absorption enhancers, the P_{app} of PEG 4000 could be increased up to a maximum value of $3.63\times10^{-6}\,cm\,s^{-1}$, a value five-fold less than that of the estimated aqueous boundary layer limiting P_{ABL}. Thus PEG permeability in the presence of the mixed micellar systems employed is primarily membrane controlled, where P_{app}/P_{ABL} is estimated to be 0.19 for the greatest value of P_{app}. In contrast P_{app} values for insulin estimated from the plasma level data were increased by up to 40-fold from mixed micellar systems, with a corresponding P_{app}/P_{ABL} value of 0.48. This suggests that paracellular transport routes do not exclusively account for insulin permeation across the intestinal epithelium.

The greater permeability of insulin might be attributed to diffusion through two parallel routes: the PEG and non-PEG

pathways. For the PEG pathway, transport is assumed to occur via passive diffusion. For the non-PEG pathway, the mechanism of transport is likely to involve simple diffusion possibly with a combination of non-specific internalization at the membrane. Ziv & Bendayan (2000) have previously reported morphological and histochemical evidence for the absorption of insulin by the epithelial cells and subsequent transfer to the circulation. These authors observed that insulin was absorbed and transferred to the blood with retention of biological activity and significant lowering of blood glucose levels. Examples of 16 to 60 amino-acid long peptides that cross cellular membranes by a receptor-independent mechanism have previously been reported (Derossi et al 1996, 1998). The findings of the present work support the existence of a transcellular pathway for insulin transport in the rat intestine.

Conclusions

The present study was conducted in order to investigate the mechanism through which insulin is absorbed by the intestinal epithelium and transferred to the systemic circulation in the perfused rat gut model. Absorption of hydrophilic solutes is classically considered as restricted to the paracellular route (Pappenheimer & Reiss 1987). The absorption of PEG 4000 when co-administered with bile salts and fatty acids with selected protease inhibitors increased up to a maximum value of 3.63×10^{-6} cm s^{-1}, a value five-fold less than its maximum possible permeability. In contrast, insulin permeability was enhanced to a greater extent, suggesting the existence of supplemental transcellular routes or pathways accessible to the insulin molecule when administered in these systems. Further definition of this transcellular pathway will contribute to a better understanding of drug delivery for potential therapeutic applications. Finally, the results of the present work suggest that markers of paracellular permeability such as PEG 4000 may result in an underestimation of the bioavailability of peptide and protein therapeutics.

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