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Insulin and didecanoyl-L-α-phosphatidylcholine: in vitro study of the transport through rabbit nasal mucosal tissue

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Summary

Transport over rabbit nasal mucosal tissue of insulin and the absorption enhancer didecanoyl-L-α-phosphatidylcholine (DDPC) from an aqueous nasal insulin preparation were studied in vitro using the Ussing chamber. The appearance of insulin at the serosal side is expressed as ‰ of the initial insulin concentration (8 mg/ml) at the mucosal side. Mean appearance rate was 0.67 ± 0.53‰ h^{-1}, equivalent to an apparent penetration coefficient (P_{app}) of 0.37×10^{-6} cm/s. Corrected for possible degradation and/or adsorption at the serosal side the value is 0.91 ± 0.79‰ h^{-1} ($P_{app} = 0.50 \times 10^{-6}$ cm/s). Addition of 20 mg DDPC/ml increased the insulin concentration a factor of only 1.3–1.5, indicating that the Ussing chamber method may not always be able to identify penetration enhancers. The concentrations of insulin and DDPC at the mucosal side between 5 and 90 min were relatively constant, the average recovery being about 85%. No transport of DDPC through the nasal tissue, using an initial mucosal concentration of 20 mg/ml, was observed. The sensitivity of the whole system was 240 μg cm^{-2} h^{-1}, or 1.2% h^{-1}. P_{app} is therefore lower than 0.33×10^{-5} cm/s. The sensitivity of the system is limited by disappearance of DDPC at the serosal side.

Introduction

At present there is a great interest in alternative routes for administration of peptides and proteins. One of these is the nasal route, however, in order to achieve the satisfactory absorption of peptides with a molecular mass greater than about 1000 Da (McMartin et al., 1987), coadministration of an absorption enhancer may be necessary. Great effort has been put into investigation of the absorption enhancing effect of various enhancers with different peptides and proteins. Possible local and systemic toxicity is related, however, to the use of enhancers. It has been shown that some enhancers, e.g., bile salts and non-ionic surfactants, disrupt the integrity of the membrane (Hirai et al., 1981). Other enhancers, such as sodium tauro-24,25-dihydro-

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fusidate (STDHF), some bile salts, polyoxyethylene 9-lauryl ether (Laureth-9) and L-α-lysophosphatidylcholine, decrease or stop the ciliary beat frequency in vitro (Gizurarson et al., 1990; Hermens et al., 1990). The other safety aspect related to possible systemic effects exerted by the enhancer is, for example, dependent on the amount absorbed.

Didecanoyl-L-α-phosphatidylcholine (DDPC) is a well known phospholipid and a natural constituent of mammalian cells. Phospholipids are also naturally occurring components in foods of vegetable and animal origin. The daily oral intake of phosphatidylcholines is about 10 mg/kg body weight (Weihrauch and Son, 1983), of which DDPC is estimated to account for 20–30 μg/kg body weight (provided that fats from other animals have the same composition as cow milk). The serum phospholipid concentration is about 1–3 mg/ml (Petersen, 1950). No data on the concentration of DDPC in serum have been reported.

Even though DDPC, as mentioned above, is an endogenous component and a natural constituent of food, it is important to know to what degree DDPC is absorbed, since a relatively high concentration of DDPC at the application site is not physiological.

Numerous enzymes in mammalian tissues have been reported to be capable of hydrolysing phosphatidylcholines and re-esterification may take place to form phospholipids with other molecular moieties (Ansell and Spanner, 1982). These conditions as well as the low plasma concentration of DDPC complicate an in vivo study of the absorption of DDPC. Therefore, the transport of DDPC as well as of insulin through the rabbit nasal mucosal tissue has been studied in vitro using the Ussing chamber technique described by Bechgaard et al. (1992).

The objective of this study was to investigate the absorption of DDPC and insulin. DDPC is a potent absorption enhancer for insulin when administered intranasally to rabbits (Hansen et al., 1988), and tested in clinical trials (Drejer et al., 1990). A further goal of this investigation was to examine local effects of the formulation by performing electrophysiological measurements.

Materials and Methods

Chemicals

Zinc-free human insulin, DDPC and insulin concentrate containing (per ml) insulin (16 mg ~ 400 IU), DDPC (40 mg), cholesterol (4 mg), fractionated coconut oil (8 mg), glycerol (32 mg), sodium phosphate (10 mM) and bicarbonate Ringer solution were provided by Novo Nordisk (Bagsvaerd, Denmark). The bicarbonate Ringer solution (GR) consisted of HPO_4^{2-} (1.6 mM), $H_2PO_4^-$ (0.4 mM), Mg^{2+} (1.2 mM), Cl^- (122 mM), Ca^{2+} (1.2 mM), K^+ (5 mM), HCO_3^- (25 mM), Na^+ (141 mM) and D-(+)-glucose (13 mM); these chemicals except glucose (May & Baker, Dagenham, U.K.) were of analytical grade and were obtained from Merck (Darmstadt, Germany). Bovine serum albumin (BSA), RIA grade was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and dissolved in GR (3 mg/ml). 5 μCi/ml ^{14}C-labelled polyethylene glycol 4000 (PEG-4000) is commercially available from New England Nuclear (Du Pont, Boston, MA, U.S.A.). Carbogen (95% O_2/5% CO_2) was supplied by Dansk Ilt & Brint fabrik A/S (Ballerup, Denmark). Scintillation cocktail (Pico-aqua) was obtained from Packard Instruments BV (Groningen, The Netherlands).

Apparatus

The acrylic Ussing chamber with accessories, Hitachi HPLC equipment for insulin analysis and scintillation counter were the same as described previously (Bechgaard et al., 1992). A Hitachi HPLC system for analysis of DDPC consisted of an L-6200 Intelligent pump, an L-4000 variable wavelength UV detector and a Rheodyne® 7125 injection valve (Berkeley, CA., U.S.A.), equipped with a 2.0 ml loop. The column was a 4 × 250 mm LiChrosorb® RP-18 (5 μm) and the guard column was a LiChroCART® 4–4 from Merck (Darmstadt, Germany). Centrifuge Labofuge® A from Heraeus Sepetech GmbH (Am Kalkberg, Germany).

Analysis

DDPC was analyzed according to an HPLC method modified from that described by Aage

Hvass, Novo Nordisk (Bagsvaerd, Denmark). The samples (50 μl) were pretreated with 50 μl 2-propanol and centrifuged for 15 min at 4000 rpm to precipitate the albumin.

The mobile phase consisted of 50% w/w 2-propanol, 0.265% w/w ammonium sulphate and 0.145% w/w orthophosphoric acid. Other conditions were: detection, at 214 nm; flow rate, 0.5 ml/min; column temperature, 50°C; injection volume, 50 μl. The retention time was about 14 min. Sample concentration was calculated on the basis of peak height relative to external DDPC standards (100 and 30 μg/ml in 0.3% w/v albumin-GR-solution (GRA)). The detection limit was about 20 μg/ml original sample and the precision about 10%.

Insulin was analyzed by the method described earlier (Bechgaard et al., 1992).

Adsorption study

The study was performed in the Ussing chamber, without membrane, thermostated at 37°C and supplied with carbogen. 2 ml GRA containing 40 μg insulin/ml and 100 μg DDPC/ml was added to the chamber. At different times, samples for DDPC and insulin analysis were collected (50 and 25 μl, respectively).

Penetration study

The tissue preparation and Ussing chamber set up were as described previously (Bechgaard et al., 1992). The tissue was preincubated for 60 min for stabilisation. GR and GRA were added to the mucosal and serosal side, respectively. In this period and during the penetration study the transepithelial potential difference (PD) and the short-circuit current (I_{sc}) were measured. After preincubation 500 μl GR was replaced with insulin concentrate, resulting in a final concentration of 8 mg insulin/ml and 20 mg DDPC/ml at the mucosal side. At different times during a 90 min period, samples for analysis of insulin and DDPC were withdrawn, 25 μl and 50 μl, respectively. Samples from both mucosal and serosal side were collected. The sample volumes were replaced.

Disappearance study

To determine the possible rate of disappearance of DDPC and insulin from the serosal side, 100 μg DDPC and 40 μg insulin were added to the serosal side immediately after the 90 min absorption study. Samples of 50 and 25 μl were withdrawn three times during a 31 min period for DDPC and insulin analysis, respectively. After the disappearance study, the tissue integrity was assessed with PEG-4000 as described by Bechgaard et al. (1992).

Calculation

The concentration of insulin and DDPC during the penetration study was corrected for dilution using the following equation:

$$Q = V_s\left(\sum_{n=1}^{n} C_{n-1}\right) + C_n V_t$$

where Q is the total amount of insulin or DDPC, V_s denotes the sample volume, V_t is the chamber volume and $C_{1,2,\ldots,n}$ represents the concentration of sample $1,2,\ldots,n$.

The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$$P_{app} = \frac{X_2}{X_1}\frac{V_2}{AT}$$

where X_2 and V_2 are the concentration and volume at the serosal side, respectively, and X_1 denotes the concentration at the mucosal side. A is the surface area of the exposed nasal mucosal tissue and T represents the time.

The rate of appearance is equal to $\alpha \times 60$ min/h, where α is the slope of the regression line (see below).

The theoretical values are calculated by carrying out linear regression on the measured values from 15 to 85 min. The theoretical value (after 90 min penetration study) is then equal to:

$$\alpha \times t - 0.1 \times \alpha \times t_{90} + 5\%o.$$

where the term $-0.1 \times \alpha \times t_{90}$ stems from the correction for the displaced volume (100 μl) at the beginning of the disappearance study.

The correction for degradation and/or adsorption is calculated from the following equation:

$$\text{Corr.}(\%/\text{h}) = (B - C)\frac{100}{1/2(A + B)} \times \frac{60}{t_b - t_a}$$

where A and B are theoretical values at 90 and 121 min, respectively, C represents the measured value at 121 min, and t_a and t_b are the times corresponding to A and B, respectively.

Results and Discussion

Adsorption of insulin and DDPC in the Ussing chamber

In general, peptides and phospholipids are very prone to adsorb to surfaces. Instability (e.g., oxidation) of phospholipids in the chamber is also possible. The disappearance of insulin and DDPC is most critical at the serosal side, where the concentration may be very low. Therefore, the study was performed at a concentration corresponding to the absorption of 0.5% of the mucosal concentration. 0.3% BSA was added to the chamber fluid to reduce adsorption and to dissolve DDPC.

During a 90 min period the decrease in insulin concentration was only about 10% (Table 1). Since the fall was initial, the disappearance of insulin is believed to be due to adsorption only. In contrast to insulin, DDPC shows a rapid initial fall followed by a slower, gradual decrease (Table 1 and Fig. 1). The change in the rate of disappearance is expected to be due to different processes. The initial fall may result from both adsorption and instability, whereas the subsequent slower decrease of about 30% h^{-1} (30 μg DDPC/h) is more likely due to instability. After 90 min, it was only possible to recover less than 20% of the initial amount of DDPC.

Penetration study

Table 2 lists data on the appearance of insulin (‰) at the serosal side relative to the initial concentration at the mucosal side. The mean rate of appearance is 0.67 ± 0.53‰ h^{-1}. The same value, corrected for possible degradation and/or adsorption (Corr.) at the serosal side, is 0.91 ± 0.79‰ h^{-1}. The observed penetration of 0.67 or 0.91‰ h^{-1} is equivalent to an apparent penetration coefficient (P_{app}) of 0.37×10^{-6} and 0.50×10^{-6} cm/s, respectively.

TABLE 1

Adsorption study: recovery of insulin and DDPC in the Ussing chamber (without tissue)

Expt no.	% insulin recovered after (min)				% DDPC recovered after (min)					
	20	40	60	90	1	5	10	30	60	90
I	91	95	83	86	78	90	71	44	32	19
II	89	93	90	89	75	67	60	40	16	nd
III	98	90	93	95	87	83	38	40	32	5
IV	86	92	83	81	90	81	78	78	36	15
V	91	93	85	90	91	78	69	61	30	29
VI	93	96	85	90	92	90	65	60	70	35
VII	91	96	85	92	100	79	69	[a]	44	18
Mean	91	94	87	89	88	81	59	54	37	17
S.D.	4	2	4	5	9	8	25	16	17	12

[a] Lost sample.

nd: non-detectable. Zero is used in the calculation for mean and S.D.

Recovery is expressed as % of the initial concentration (40 μg insulin/ml and 100 μg DDPC/ml) in the chamber.

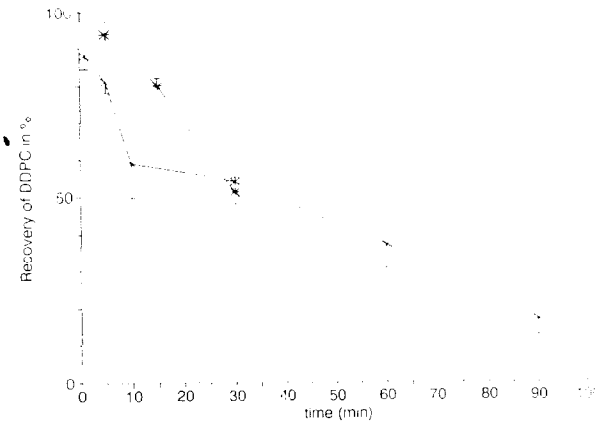

Fig. 1. Recovery (% (mean ± SE; initial concentration, 100 μg/ml) of DDPC in the Ussing chamber. (○) ($n = 8$) Without tissue; (●) ($n = 7$) serosal recovery after addition of 100 μg DDPC following the 90 min penetration study. Dashed lines represent presumed course.

TABLE 2

Penetration study: appearance of insulin. Appearance of insulin at at the serosal side

Expt. no.	Serosal side (recovery ‰ at min)								Appearance rate	
	15	35	55	70	85	96	106	121	‰/h	(Corr.) [b]
I	0.04	0.12	0.31	0.39	0.48	5.79	4.88	5.61	0.39	0.42
II	0.04	0.05	0.09	0.13	0.17	5.29	4.55	5.29	0.11	0.11
III	0.04	0.22	0.39	0.59	0.76	5.96	5.98	6.10	0.62	0.65
IV	0.21	0.90	1.71	2.32	2.23	6.06	5.89	6.99	1.90	2.71
V	0.11	0.19	0.34	0.57	0.69	4.78	4.84	4.83	0.52	0.73
VI	0.00	0.08	0.24	0.37	0.46	4.50	4.50	4.63	0.41	0.58
VII	[a]	0.47	0.61	0.89	1.01	4.94	4.75	5.00	0.68	0.97
VIII	0.07	0.29	0.46	0.74	0.87	4.60	4.59	4.70	0.70	1.07
Mean	0.07	0.29	0.52	0.75	0.83	5.24	4.59	5.39	0.67	0.91
S.D.	0.07	0.28	0.50	0.68	0.62	0.64	0.62	0.81	0.53	0.79

[a] Not measured.

[b] Including the correction for degradation and/or adsorption.

Insulin appearance is expressed in ‰ of the initial mucosal concentration (8 mg/ml) at different times (min) during the experiment. At 90 min 5‰ insulin is added to the serosal side to estimate the correction for degradation.

The mean rate of appearance of insulin without enhancer was found to be 0.44 ± 0.34‰ h^{-1} (0.73 ± 0.42‰ h^{-1}, Corr.) by Bechgaard et al. (1992). Therefore, the addition of 2% DDPC apparently increases the penetration by a factor of 1.3–1.5, only. However, this is not significant taking the experimental variation into account. Carstens et al. (1992) found that 0.5% DDPC increased the penetration of insulin in vitro by a factor of 3. The penetration of insulin without DDPC was even higher than that determined in the present paper by a factor of 2. Possible reasons for this discrepancy may be that the above-mentioned authors used ^{125}I-insulin, measured unspecific radioactivity, and only corrected for degradation fragments of molecular mass below about 1000 Da. In addition, they used a 14 000-fold lower mucosal concentration, which is relatively more degradable as the peptidases may not have been saturated (Gizurarson and Bechgaard, 1991).

Despite the low rate of appearance of insulin at the serosal side, the mucosal concentration dropped about 20% (Table 3) within 5 min, and was relative stable thereafter. Since the adsorption study only demonstrated a 10% initial fall in insulin concentration and since this decrease was from a much more diluted solution, the results were surprising. The presence of tissue may be the reason for the greater decrease. Since the decrease is only initial, it is not expected to be due to enzymatic degradation. Gizurarson and Bechgaard (1991) have also shown in vitro that the enzymatic degradation of insulin is relatively

TABLE 3

Penetration study: recovery of insulin and DDPC at the mucosal side

Expt. no.	% insulin recovered after (min)				% DDPC recovered after (min)			
	5	25	45	90	10	30	65	90
I	62	73	86	82	66	75	79	88
II	96	95	90	86	80	83	80	92
III	77	75	69	74	74	77	76	80
IV	75	79	[a]	79	86	94	99	101
V	83	89	92	87	93	90	88	80
VI	76	86	87	88	87	99	84	94
VII	79	91	92	93	93	107	103	120
VIII	72	80	79	79	70	73	74	83
Mean	78	84	85	84	81	87	85	92
S.D.	10	8	8	6	10	12	11	13

[a] Lost sample.

Recovery is expressed as % of the initial concentration (8 mg insulin/ml and 20 mg DDPC/ml) in the chamber.

small at high concentration. The absence of BSA at the mucosal side in order to prevent adsorption to the chamber could represent another explanation.

The appearance of DDPC at the serosal side was below the detection limit of about 20 μg/ml, equivalent to less than 0.1% of the concentration at the mucosal side. At the mucosal side the extent of the decrease in concentration only amounted to about 15% (Table 3). The disappearance study showed a 50% fall in DDPC concentration at the serosal side after 30 min, equivalent to 100 μg/h (Fig. 1); the decrease appears to be linear with respect to time. As instability of DDPC in the chamber may account for about 30 μg/h, approx. 70 μg/h may undergo degradation by enzymes from the 0.5 cm^2 surface area of serosal tissue (140 μg DDPC h^{-1} cm^{-2}).

A rate of transport above 120 μg DDPC/h (disappearance + detection limit) is therefore detectable. 120 μg DDPC/h is equivalent to 0.6% of the initial concentration at the mucosal side. As the surface area amounted to 0.5 cm^2, the transport of DDPC was less than 240 μg h^{-1} cm^{-2} or 1.2%/h (P_{app} lower than 0.33×10^{-5} cm/s).

Electrical properties of the tissue

After preincubation the electrical properties of the tissues showed some intervariation. The short-circuit current (I_{sc}) was 71 ± 35 $\mu A/cm^2$ and the transepithelial potential difference (PD) was 2.5 ± 0.7 mV (serosal side positive relative to the mucosal side).

The PD was about 50% lower than that reported in our previous work (Bechgaard et al., 1992), however, I_{sc} was of the same magnitude. The only difference in the study design was the concentration of BSA (0.1 vs 0.3% w/v).

Bechgaard et al. (1992) have shown that rabbit nasal mucosal tissue can remain viable for more than 12 h in the Ussing chamber under the given circumstances. Addition of 2% DDPC to the mucosal side resulted in I_{sc} and PD falling to zero within less than 15 min. This drop may indicate a local effect on the tissue cells, nevertheless, it is difficult to predict whether this could occur in vivo, where the formulation is diluted due to mucosal secretion, and where the tissue 'repair' processes are normal. At 0.1 and 0.5% DDPC this effect on the electrical properties is reversible (Carstens et al., 1992). Similar results obtained for other enhancers (Wheatley et al., 1988; Gizurarson et al., 1990) have demonstrated that an insulin preparation containing 20 mg DDPC/ml has no significant influence on the mucociliary transport rate in vitro.

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