Permeability of Insulin Entrapped in Liposome through the Nasal Mucosa of Rabbits

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The permeability of liposome entrapping insulin through the nasal mucosa of rabbit has been studied and compared with the permeability of insulin solution with or without pretreatment by sodium glycocholate (GC). Insulin entrapped in liposome was not detected in the receiver cell using the diffusion cells with the nasal mucosa. On the other hand, permeability of insulin entrapped in liposome increased after the pretreatment of GC. The phospholipids which result from liposomes, were not observed in the receiver. Also, the GC remaining in the nasal mucosa was measured. Considering the mechanism of permeation of insulin entrapped in liposome through the nasal mucosa, the GC remaining in the nasal mucosa may cause the lysis of liposomes.

Keywords permeability; liposome; nasal mucosa; insulin; partition

Recently, intranasal administration has been investigated as a new route for systemic peptide delivery.[1,2] The use of liposomes as a transmucosal therapeutic system has many advantages, for example, protection of the drug from degradation by peptidase on the mucosa,[3] maintenance of a high concentration at the site of administration and facilitation of their absorption through the mucosa.

However, use of liposomes for topical application has received little attention. Ganesan *et al.*,[4] Meisner *et al.*, [5] and Knepp *et al.*,[6] reported the potential use of liposomes in topical applications for the skin. These reports indicate that liposomes facilitate the passage of drugs through the skin. However, the mechanism by which drugs traverse the skin is not yet understood.

We have investigated the influence of the permeability of liposome entrapping insulin to determine the extent to which liposomes promote the permeation of insulin through the nasal mucosa, and to determine if liposomes themselves pass through the nasal mucosa.

Experimental

Materials L-α-Dipalmitoylphosphatidylcholine (DPPC), cholesterol (C), dicetyl phosphate (DCP) and crystalline bovine pancreas insulin (24.5 U per mg, crystalline, zinc content, approx. 0.5%) were purchased from Sigma (St. Louis, MO, U.S.A.). DPPC was judged as pure on the basis of normal silica-gel thin-layer chromatography criteria. Sodium glycocholate (GC) was purchased from Nacalai Tesque. Inc. (Kyoto, Japan). All other chemicals were of analytical grade and used as received. Water used throughout the experiments was membrane-filtered deionization water (Milli-Q, Nihon Millipore Kogyo, K.K., Japan).

Preparation of Liposomes Liposomes were prepared according to a standard method.[7] A mixed solution of DPPC (35 μmol), C (35 μmol) and DCP (1 μmol) in $CHCl_3$ solution was transferred to a 50 ml round-bottom flask and the solvent was evaporated. After 15 h *in vacuo*, the dried lipid film was swollen in 2.5 ml of phosphate-buffered saline (PBS, 137 mM NaCl/2.6 mM KCl/6.4 mM Na_2HPO_4/1.4 mM KH_2PO_4; pH 7.4). For experiments involving insulin, PBS containing 20.97 U/ml insulin was used. This dispersion containing swollen lipids was kept above the phase transition temperature (42 °C), and was mixed by vortexing for 10 min followed by sonication for 10 min in a bath-type sonicator (Honda Electronics W220R, Japan). The liposome suspension was allowed to stand for 16 h to complete hydrations of the lipids, and diluted to a final concentration of about 28.4 μmol lipid per ml. The liposome suspension was extruded through the 1 μm membrane and successively through the 0.4 μm pore diameter (Nuclepore, Nomura Micro Science Co., Japan). The liposomes with entrapped insulin were fractionated by passing 1 ml of liposome suspension over a column (1.8 × 35 cm) of Sephadex G-50 (Pharmacia, Uppsala, Sweden) with the PBS. Each fraction volume was 4.5 ml.

Nasal Mucosa Permeation Studies The nasal mucosa used in these experiments was obtained from male Japanese white rabbits (Saitama Experimental Animal Supply Co., Japan; body wt., 2.5—3.0 kg). Nasal mucosa was obtained from the anterior orbits of the junction of the nasal bone with the dosal pariental cartilage, as previously described.[8] After being rinsed in saline solution and then distilled water, a piece of the nasal mucosa was mounted as a flat sheet on a circular window with a 0.503 cm^2 area. The thickness was measured by a thickness gauge with an accuracy of ±0.001 mm. After mounting the membrane in the diffusion cell, it was immersed in a constant temperature waterbath of 37 °C.

The donor solution was in contact with the mucosal surface which is the ciliated epithelial surface lining the nasal cavity and the receiver solution was in contact with the serosal surface which is the side attached to the nasal cartilage. A 7 ml aliquot of insulin in PBS solution or liposomes in PBS was used as the donor solution. The initial insulin activity in the donor solution was 225 mU/ml for insulin solution, 157 mU/ml for insulin in liposome, respectively. A 7 ml aliquot of PBS solution was used for the receiver solution.

Pretreatment with GC is as follows. After the membrane was clamped between the two chambers, the mucosal surface of the nasal mucosa membrane (donor side) was bathed in a solution of GC (10 mM) for 5 min and the solution was well stirred. After the mucosal surface was rinsed, then bathed with distilled water and the water discarded, the donor solution was then added. Also, a fresh receiver solution was used. A 300 μl sample was withdrawn with a micropipette from the receiver cell at designated times over a 4 h period. The same amount of saline was added to the receiver solution.

From the plots of the concentration of permeant appearing in the receiver cell with time, the permeability coefficients (P) were calculated. The flux of mass from the donor cell is assumed to be equal to the appearance rate of mass in the receiver.

$$J = APC_D = V \cdot dc/dt \quad (1)$$

where J is the flux, A is the area of the nasal mucosa, C_D and c are the drug concentrations in the donor and receiver cell, respectively, V is the volume of the receiver cell (7 ml) and t is time. Experimentally, C_D does not appreciably change during the experimental period; therefore, sink conditions in the receiver cell essentially prevail. Consequently, the permeability coefficient is determined by

$$P = (V/AC_D)(dc/dt) \quad (2)$$

Measurement of GC in the Nasal Mucosa After the nasal mucosa was pretreated with GC, it was homogenized with 5 ml of the rinse solution using a homogenizer (Ultra-turrax®, Kubota Trading Co., Ltd., Japan). The homogenates were centrifuged at 9000 × *g* at room temperature for 10 min to remove cellular and nuclear debris. Next, the resultant supernatants were freeze-dried, and then 300 μl of distilled water was added to them to measure the concentration of GC using Enzabile 2 (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan).

Analysis of Lipid and Insulin The lecithin concentration was measured using a commercially available kit (phospholipids B-test; Wako Pure Chemical Co.). The insulin activity was determined by an enzyme immunoassay method, with a commercially available kit (Insulin EIA Kit, Dainabot Co., Ltd., Japan) as described previously.[9]

***In Vitro* Release Rates of Insulin Entrapped in Liposomes** 5 ml of liposome suspension after gel-filtration was packed in a bottle. Exactly 500 μl samples were withdrawn at designated times from the sample

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bottle and centrifuged for 30 min at 25000 × *g*. The supernatant liquid was used to measure insulin activity.

Determination of Partition of Insulin Isopropyl myristate was used as the oil phase and water or PBS was used as the aqueous phase. The water or PBS and isopropyl myristate phases were equilibrated with each other prior to the commencement of the partitioning experiment. Isopropyl myristate (2 ml) was added to water or PBS (2 ml) containing 30 mU of insulin in a test tube. Only insulin solution is used for control since insulin is adsorbed on the test tube. The partition coefficients were obtained by measuring the insulin activity in the aqueous phase after incubation (2, 4 h) at 37 °C. The permeability coefficient, P, can be represented as Eq. 3,

$$P = f \cdot D \cdot K/h \quad (3)$$

where f is the membrane constant, D is the diffusion coefficient, K is the partition coefficient and h is the thickness of the nasal mucosa.

Results

Nasal Mucosa Permeation Studies Figure 1 shows the appearance of insulin in the receiver cell when the donor is provided with an insulin solution or liposome suspension with or without the pretreatment by GC. GC was selected as an enhancer since it was reported that GC is a very effective enhancer for nasal absorption of peptides, such as insulin and interferon.[10,11] The insulin activity was detected to be very low in receiver cells without pretreatment by GC for both insulin solution and liposome suspension. However, the insulin permeation through the nasal mucosa was increased by pretreatment with GC for both insulin solution and liposome suspension.

The permeability coefficients of the insulin solution and liposome suspension are shown in Table I. The permeability coefficients were obtained from the data shown in Fig. 1 using Eq. 2. The data at pH 2 and 5.4 were obtained from a previous paper.[8] The pH of 2 or 5.4 for the insulin solution was adjusted using hydrochloric acid. Insulin in saline solution at pH 5.4 did not permeate even with pretreatment by GC and insulin in PBS at pH 7.4 hardly permeated without pretreatment by GC. The permeability of insulin solution at pH 7.4 is lower than that at pH 2.0 with pretreatment by GC. Insulin entrapped in liposome did not permeate without pretreatment by GC but permeated with pretreatment by GC. The permeability of insulin in liposome suspension at pH 7.4 with pretreatment increased to similar values with that at pH 2.0 without pretreatment.

Residual insulin activities in donor cells after permeation measurement of insulin solution and liposome suspension were measured, as shown in Fig. 2. In insulin solution, when the insulin activity in donor cells was 225 mU/ml, residual insulin activity was 86.0 ± 32.0 mU/ml without pretreatment, and 132.1 ± 17.1 mU/ml with pretreatment. In liposome suspension, residual insulin activity with pretreatment was 123.0 ± 11.0 mU/ml though insulin activities without pretreatment were the same values as the starting one.

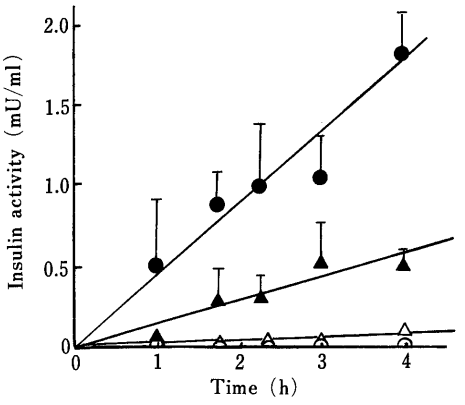


Fig. 1. Insulin Activity *versus* Time in the Receiver Cell at 37 °C

Error bars represent ±S.D. ($n=3$). △, insulin solution (225 mU/ml); ▲, insulin solution with pretreatment by GC (225 mU/ml); ○, insulin in liposome (356 mU/mg phospholipid, 157 mU/ml); ●, insulin in liposome with pretreatment by GC (290 mU/mg phospholipid, 225 mU/ml).

TABLE I. Permeability Coefficients of Insulin Across the Intact Excised Nasal Mucosa of Rabbits with and without Pretreatment of 10 mM Sodium Glycocholate

pH	Donor (insulin)	Pretreatment	$P \times 10^6$ (cm/s) mean ± S.D. ($n=3$)
2.0	Solution	−	8.38 ± 1.25 a)
2.0	Solution	+	22.4 ± 1.54 a)
5.4	Solution	+	0.00 a)
7.4	Solution	−	0.95 ± 0.18
7.4	Solution	+	2.35 ± 1.09
7.4	Liposome	−	0.36 ± 0.19
7.4	Liposome	+	7.15 ± 2.57

a) Reference 8.

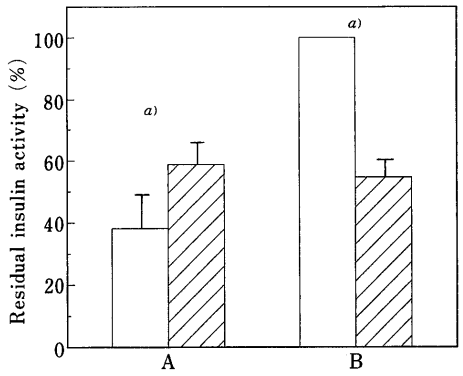


Fig. 2. Residual Insulin Percents in Donor Solution after Permeation Measurement of Insulin and Liposome Suspension at 37 °C

A, insulin solution; B, liposome suspension, (unfilled bar) without GC; (hatch bar) with GC, error bars represent ±S.D. ($n=3$). Statistical comparisons were performed by Student's t-test: a) $p<0.01$, *vs.* control.

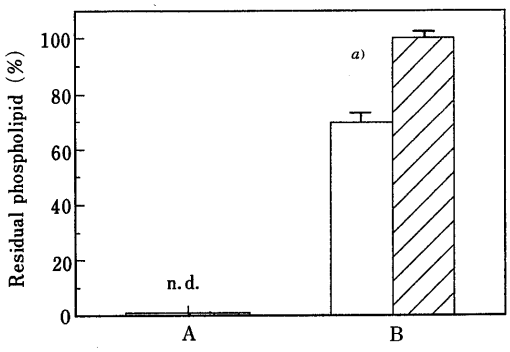


Fig. 3. Residual Phospholipid Activity in Donor Solution after Permeation Measurement of Insulin and Liposome Suspension at 37 °C

A, insulin solution; B, liposome suspension; (unfilled bar) without GC; (hatch bar) with GC, error bars represent ±S.D. ($n=3$). Statistical comparisons were performed by Student's t-test; a) $p<0.01$ *vs.* control.

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TABLE II. Partition Coefficient ($\log K$) of Insulin (30 mU, Mean; $n=2$) in Isopropyl Myristate/Water or PBS (pH 7.4) at 37 °C as a Function of Time

	Time (h)	
	2	4
Water (pH 5.5)	1.65	1.76
PBS (pH 7.4)	−0.764	−0.619

$\log K = \log[(C - C_w)/C_w]$; C and C_w are the insulin activity in aqueous phase before and after incubation, respectively.

Residual phospholipid in donor cells after permeation measurement of insulin solution and liposome suspension was measured, as shown in Fig. 3. Phospholipid in both the donor and receiver cells was not detected in the case of the insulin solution. This means that the nasal mucosa did not release the phospholipid component.

In liposome suspension, the residual concentration of phospholipid without pretreatment by GC was significantly decreased (44.13 ± 2.13 mg/dl) in the donor cell when the concentration of phospholipid in the donor cell was 63.26 ± 3.71 mg/dl. On the other hand, after pretreatment by GC, the residual concentration of phospholipid was the same as that of the initial one.

***In Vitro* Release of Insulin from Liposomes** The release of insulin from liposome *in vitro* was very slow. Insulin was not released from the liposome *in vitro* at 37 °C for 4 h without GC.

Partition of Insulin Table II demonstrates the effect of incubation time on isopropyl myristate/water or PBS partition coefficient ($\log K$) of insulin. The value of $\log K$ at pH 7.4 was lower than at pH 5.5 and increased with incubation time.

Discussion

Characterization of Liposomes Our system produces 0.23—0.32 μm multilamellar liposomes. They are composed of DPPC, C and DCP (molar ratio, 7:7:0.2) and have a high capture efficiency for insulin (1.58—1.94 mmol insulin/mol phospholipid). Previously we reported that insulin entrapped in liposomes did not affect the surface potential of liposomes.[9] The liposome with entrapped insulin charged negatively in PBS at pH 7.4 because DCP in liposome was dissociated.

Nasal Mucosa Permeation Studies Ganesan *et al.*,[4] reported that highly polar glucose in liposome is poorly available for the transport of mice skin but a lipophilic drug like progesterone in liposome passed through the skin. However, Fig. 1 shows that insulin in liposome did not penetrate through the nasal mucosa since insulin is more lipophilic than glucose, but not so lipophilic as progesterone.

Table I shows that insulin permeated through the nasal mucosa at pH 2.0 without pretreatment by GC because insulin forms a monomer[12] and is positively charged at this pH.[13]

Insulin at pH 5.4 did not permeate with pretreatment by GC. Insulin forms a hexamer or octamer and is not charged at pH 5.4 because the isoelectric point of insulin is about 5.4 (more exactly, it is from 5.3 to 5.35). The permeability of insulin solution at pH 7.4 ($2.35 \pm 1.09 \times 10^{-6}$ cm/s) is lower than that at pH 2.0 (22.4×10^{-6} cm/s) with pretreatment by GC. This difference is caused by the fact that the ratio of a monomer of insulin is small and insulin is charged negatively at pH 7.4 since the nasal mucosa was also negatively charged.[11]

Permeability of insulin entrapped in liposome ($0.36 \pm 0.19 \times 10^{-6}$ cm/s) is smaller than that of the free one at pH 7.4 ($0.95 \pm 0.18 \times 10^{-6}$ cm/s). In these cases, liposome is negatively charged because of containing DCP at pH 7.4. After pretreatment by GC, permeability of insulin entrapped in liposome ($7.15 \pm 2.17 \times 10^{-6}$ cm/s) is higher than that of insulin solution ($2.35 \pm 1.09 \times 10^{-6}$ cm/s). It is considered that GC may work on liposome and the activity of insulin from liposome on the nasal mucosa may be high locally.

It is well known that bile salt has many roles as an enhancer of nasal absorption. Some possible roles of GC are as follows: Gordon *et al.*,[14] reported that GC was absorbed in the mucosa and modified the mucosa; Hersey and Jackson[15] reported that epithelial cells, which were the barrier to permeation, were drawn out by pretreatment with bile salts; Hirai *et al.*,[10] Hayakawa *et al.*,[16] reported that bile salt inhibits peptidase in the nasal mucosa, and also bile salt may prevent self-association of insulin as sodium salicylate.[17]

Comparing the insulin solution and liposome suspension, the liposome had permeated more effectively after pretreatment by GC. Liposome facilitates the passage of insulin through mucosa after pretreatment by GC. However, it is not known whether the liposomes traverse the mucosa or not. Therefore, the phospholipid of liposome in the receiver cell was measured after the permeation experiment.

As shown in Fig. 1, the penetration of insulin with pretreatment increased more than that without pretreatment by GC. Therefore, if the absorption of insulin in the nasal mucosa is the same between them, residual insulin activities in donor cells with pretreatment by GC must be lower than that without pretreatment by GC. However, after GC pretreatment, the residual insulin activity in insulin solution increased and that in liposome suspension decreased. The experimental result about residual insulin activities in insulin solution contradicts this fact. The explanation may be that GC remaining in the nasal mucosa inhibits the peptidase[17] and makes insulin stable in the nasal mucosa thus preventing formation of the enzyme–insulin complex.[18]

Figure 3 suggests that DPPC molecules are so locked into the liposomal structure that no appreciable free phospholipid exists to partition into the mucosa and permeate after pretreatment by GC. The decrease in the concentration of phospholipid in the donor cell might be due to absorption into the nasal mucosa without the pretreatment by GC. Phospholipid in the receiver cell was not detected in the case of the liposome suspension with pretreatment by GC. This result clearly shows that the liposomes do not pass intact through the mucosa, but they do induce a remarkable enhancement in the permeation of insulin entrapped in them.

Partition of Insulin From the result of the nasal mucosa permeation, the permeability of insulin solution at pH 5.4 is much lower than at pH 7.4, as shown in Table I. For

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insulin, equilibrium was attained within 4 h. It is often advisable to allow the equilibration time to be as long as possible. In this case, the use of long incubation periods changes the stability of insulin. The 4-h optimal equibration time for insulin is considered. From the partition experiment of insulin (Table II), the values of $\log K$ in PBS (pH 7.4) were smaller than ones in water (pH 5.5). This result corresponds in that insulin is not charged around pH 5.4 and charged at pH 7.4. However, insulin aggregates around pH 5.4, therefore, permeability of insulin at 5.4 was lower than that at pH 7.4, as shown in Table I.

By using Eq. 3 in insulin solution at pH 7.4, the value of fD is estimated 7.52×10^{-8} cm^2/s when h is 0.019 cm (measured), P is 0.95×10^{-6} cm/s (Table I) and $\log K$ is -0.619 (Table II). This value is in the expected range for the diffusion coefficient of a molecular such as insulin.

GC in the Nasal Mucosa The permeation of insulin is now interpreted by three mechanisms. First, liposome with entrapped insulin releases insulin and then the released insulin permeates. Second, liposome with entrapped insulin penetrates through the nasal mucosa. Third, liposome with entrapped insulin was broken at the surface of the nasal mucosa, and liberated insulin which is localized in high concentration in the nasal mucosa, is effectively transferred.

Experimentally, insulin was released very slowly from the liposome *in vitro* at 37 °C for 4 h and could not be detected. Liposome was not transferred to the nasal mucosa since the phospholipid was not detected in the receiver cell. Therefore, the first and second mechanisms are not acceptable. GC may not only work to stabilize the insulin activity but may also have some roles in transferring insulin entrapped in the liposome through the nasal mucosa.

To elucidate the role of GC to liposome and nasal mucosa, the uptake amount of GC in the nasal mucosa was measured after pretreatment by GC. It was thus found that the amount of GC remaining in the nasal mucosa was $8.68 \pm 3.54 \times 10^{-7}$ mol GC per g nasal mucosa. After the pretreatment of 10 mM GC solution for 5 min, less than 10% of GC (10 μmol/ml) absorbed in the nasal mucosa. It is assumed that the molar ratio of GC to insulin is very high (about 567) when the insulin in liposome (225 mU/ml, 1.53×10^{-3} μmol/ml) in a donor cell comes in contact with GC in the nasal mucosa. Hayakawa *et al.*,[16] reported that at insulin concentrations in the nasal homogenates lower than 50 μM, 2% GC reduced the rate of insulin proteolysis (the molar ratio of GC to insulin is 205). By contrast, at 100 μM insulin concentration, GC accelated insulin proteolysis (the molar ratio of GC to insulin is 410). In our data, the molar ratio of GC to insulin was 567 but GC remaining in the nasal mucosa may inhibit the insulin proteolysis. The difference may be because the nasal mucosa in the permeation experiment was not homogenates and insulin was entrapped in liposome. Therefore, the molar ratio GC to insulin was higher than the 410 that was the reported molar ratio GC accelated insulin proteolysis. In our experiment, GC may enhance the nasal transport of insulin.

Conclusion

From the result of permeability of liposomes entrapping insulin through the nasal mucosa, it is concluded that the GC remaining in the nasal mucosa caused the lysis of liposome which came to the surface of the nasal mucosa and caused the release of insulin from the liposome, and the high activity of insulin on the mucosa surface resulted in the effective permeation of insulin through the mucosa.

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