Influence of Liposomes on Tryptic Digestion of Insulin. II

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The mechanism of enhancement of tryptic digestion of insulin by empty liposomes was studied using HPLC analysis, gel filtration (insulin binding to the liposome and lipid transfer to the insulin) and an electrokinetic study using the zeta meter (trypsin binding to the liposome). Soybean phosphatidylcholine (PC), phosphatidic acid (PA) [PA/PC = 1/9] and stearyl amine (StA) [StA/PC = 1/9] were used as neutral, negatively charged and positively charged liposomes, respectively.

Tryptic digestion of insulin was enhanced by reducing the liposome size from 150 to 40 nm when neutral empty liposomes were used. The amount of insulin bound to neutral empty liposomes increased on reducing liposome size. Nevertheless, no strong evidence of trypsin binding to neutral empty liposomes was obtained.

The amount of liposome-bound insulin was greater for PC than StA/PC and PA/PC, and the amount of lipids transferred to insulin decreased in the following order; StA/PC > PA/PC > PC. These findings suggest that the positively charged liposome did not enhance tryptic digestion, because insulin was protected from tryptic digestion by surrounding lipids from positively charged liposomes (StA/PC). Trypsin bound to the PA/PC liposomes, but not to the PC or StA/PC liposomes.

Keywords insulin; trypsin; binding; liposome; digestion

Liposomes have been studied as drug carriers for many years[1] and they are useful for protecting drugs from degradation in the blood. Protein and peptides are easily degraded by enzymes in the blood or gastrointestinal tract. Recently, liposomes have been applied in the use of insulin.[2-5] The interaction of proteins and liposomes has been studied to investigate why liposomes are unstable in blood[6-11] and to study the mechanism of enzymatic reactions on the surface of the membrane.[12-14]

Previously, we reported that the digestion of insulin by trypsin was enhanced by empty liposomes.[15] We concluded that the digestive reaction between insulin and trypsin occurs on the surface of these empty liposomes. The many unsuccessful attempts to use liposomal insulin clinically may be due to the enzymatically digested insulin leaking out of the liposomes.

Proteins with a high moiety of α-helix in their molecule such as apoproteins[16,17] or serum albumin[18,19] have a high affinity for liposomes. Recently, the uptake of liposomes by the reticuloendothelial system (RES) has been seen to depend on the opsonization.[10,20-22] Previously, we reported that a larger number of 20 nm liposomes, containing α-tocopherol as a model drug, distributed to the liver than 80—200 nm liposomes.[23] These phenomena may depend on the opsonization by serum components.

Wiessner and Hwang reported that insulin was bound to the surface of the liposomes after gel filtration chromatography.[24] Law *et al.* described the electrokinetics of the interaction between microparticles (liposomes and latexes) and bovine serum albumin using the zeta meter.[18] Insulin may act like these proteins to induce opsonization. Since a major part of the insulin molecule consists of α-helix (42%), we examined the interaction between insulin and liposomes. Here, we describe the binding of insulin to empty liposomes depending on their size or charge, and the electrokinetics of the interaction between trypsin and the empty liposomes.

Materials and Methods

Materials Bovine insulin was purchased from Sigma Ltd., porcine trypsin (2.5% solution) from Gibco Ltd., and soybean phosphatidylcholine (PC; Epikuron 200) was purchased from Nikko Chemicals. Phosphatidic acid (PA) and sodium glycocholate were purchased from Sigma Ltd. Stearylamine (StA) and TritonX-100 were purchased from Wako Pure Chemical Ltd. All materials were used without any further purification.

Tryptic Digestion of Insulin Two mg of insulin was dissolved in 1 ml of 0.02 N HCl, and then diluted to give a solution containing 0.4 mg/ml of insulin with 0.05 M pH 7 phosphate buffer. This was used as the stock solution. Tryptic digestion of insulin was performed under the following conditions;

Insulin; 1.7×10^{-5} M, trypsin; 4.3×10^{-6} M, phospholipids; 0, 5.1×10^{-3} M, in 0.01 M pH 7 phosphate buffer, at 37 °C.

At appropriate intervals, samples were collected and stored on ice. Then 0.5 ml aliquot of 5% TritonX-100 and 0.5 ml of 0.2% sodium glycocholate were added to 1 ml of each sample and analysis by high performance liquid chromatography (HPLC) was carried out immediately. TritonX-100 was used as a detergent for the liposomes while sodium glycocholate was used to prevent insulin adsorption to the vessel walls and also as the internal standard of HPLC analysis.

Preparation of Empty Liposomes Multi-unilamellar vesicles (MLV) were prepared as described by Bangham *et al.*[25] Large unilamellar vesicles were prepared from MLV by extrusion techniques (LUVET, vesicles are unilamellar or oligolamellar). The MLV suspension was passed ten times through a polycarbonate membrane filter (Nucleopore, pore size 0.05—0.3 μm, Nomura Micro Sciences Ltd.) to size the liposomes as described by Hope *et al.*[26] We used three lipid compositions; PC only, PA/PC (1/9 mol), or StA/PC (1/9 mol).

HPLC Analysis The HPLC system consisted of a Hitachi 655-15 liquid chromatography, a Hitachi variable-wavelength UV monitor, and a Shimadzu CR 2AX Chromatopac. The column was a 3.9 × 150 mm, Waters Nova-Pak C18, 4 μm. The mobile phase consisted of the mixture of acetonitrile and 0.01 M phosphoric acid–sodium perchlorate solution (1 : 2). Triton X-100 was added to the mobile phase (0.01% w/v). Insulin was detected at 210 nm and sodium glycocholate was used as the internal standard.

Measurement of Particle Size Liposome size was determined using a dynamic laser light scattering instrument (Model DLS-700, Otsuka Electronics Ltd.).

Binding Studies of Insulin to Empty Liposomes Insulin was allowed to bind to liposomes by incubating various types of liposome, containing 5.1×10^{-3} M of lipids, with 1.7×10^{-5} M of insulin in 0.01 M pH 7 phosphate buffer at 25 °C for 5 min. After incubation, the mixture (0.5 ml) was immediately subjected to molecular sieve chromatography on a Sepharose CL-4B (Pharmacia) column (1.5 × 20 cm) equilibrated with ten-fold diluted Dulbecco phosphate buffered saline without Mg^{2+} and Ca^{2+} at 25 °C. The eluate was collected in glass test-tubes using a fraction collector (1 ml/fraction). A portion (0.4 ml) of each fraction was transferred to a

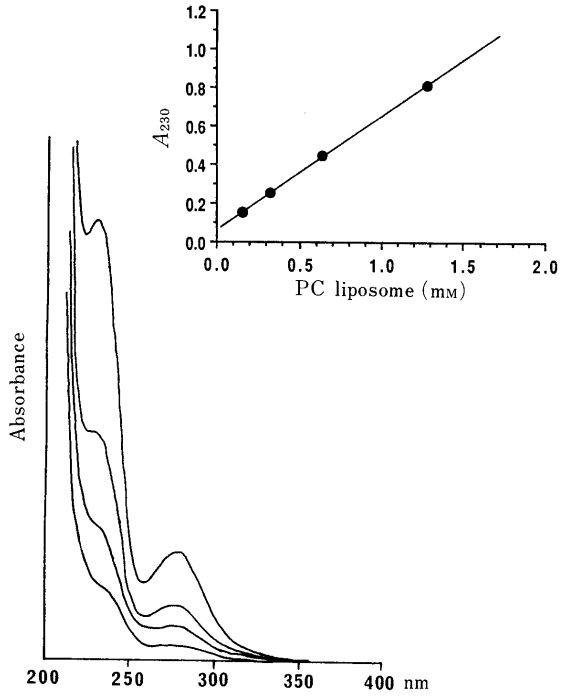

Fig. 1. UV Spectrum of PC in EtOH–Water (1.5 : 0.5, v/v) Solution and Calibration Curve at 230 nm

The line represents a linear least-squares analysis. PC: 0.16, 0.32, 0.64, 1.28 mM.

glass test-tube, and then 0.4 ml of water and 50 μl of Bio-Rad protein assay dye reagent (Bio Rad) were added. The elution profile was obtained by measuring the absorbance at 595 nm.

Liposome fractions (0.4 ml/fraction) were collected in the same test tube and then 3 ml of ethanol and 1 ml of ether were added. The same procedure was performed for the insulin fractions. Organic solvents containing buffer solution were dried under vacuum. Then 3 ml of ethanol and 1 ml of ether were added to the test tube, and the suspension was centrifuged (3000 rpm–10 min) to separate the insulin precipitate from the lipid solution. This separation procedure was performed twice. The insulin precipitate was dissolved with 0.1 ml of 0.02 N HCl, and then 50 mM pH 7 phosphate buffer solution and 50 μl of Bio-Rad protein assay dye reagent (Bio Rad) were added to the protein solution for determination of the insulin concentration. The lipids in the supernatant were dried under vacuum and reconstituted with 0.5 ml of distilled water and 1.5 ml of ethanol. The lipid concentration was determined by measuring absorption at 230 nm using the calibration curve obtained with initial empty liposomes (Fig. 1).

Binding Studies of Trypsin to Liposomes Trypsin was allowed to bind to liposomes by incubating various types of liposomes containing 6.4×10^{-4} M of lipids with 0.5—5 mg/ml of trypsin in 0.05 M pH 7 phosphate buffer at 25 °C for 5 min. After incubation, the zeta potential of the mixture was measured. Measurement of the zeta potential was performed in 0.05 M phosphate buffer, because reproducibility was poor in 0.01 M phosphate buffer.

Measurement of Zeta Potential Zeta potentials were determined in 0.05 M pH 7 phosphate buffered solution by electrophoretic measrements using a dynamic laser light scattering detection electrophoretic apparatus (ELS-800, Otsuka Electronics Ltd.).

Results

Effect of Empty Liposomes on Tryptic Digestion of Insulin We previously reported that tryptic digestion of insulin was enhanced by decreasing the size of the empty liposome (from 2 to 0.1 μm) when the same amount of lipid was used for liposomes.[15] We examined the enhancement of tryptic digestion by empty liposomes for a liposome size ranging from 40 to 150 nm. Figure 2 shows the residual insulin after tryptic digestion at 37 °C. Tryptic digestion of insulin increased with progressively decreasing liposome size as we previously reported.[15]

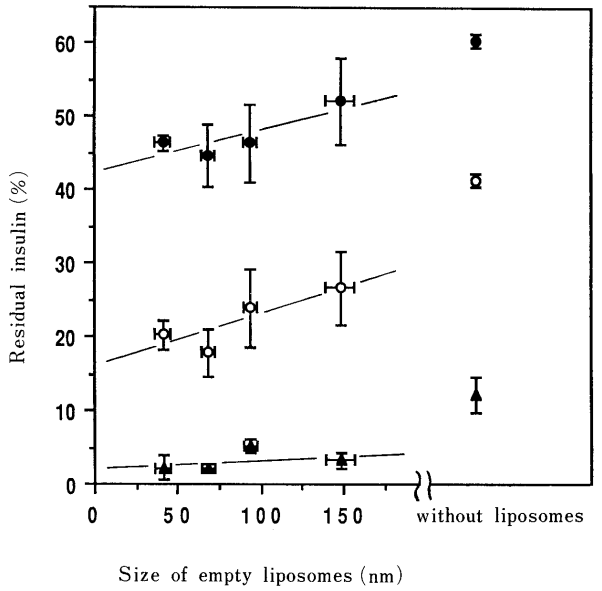

Fig. 2. Effect of Empty PC Liposomes on Tryptic Digestion of Insulin

●, after 15 min; ○, after 30 min; ▲, after 60 min. Insulin: 1.7×10^{-5} M; trypsin: 4.3×10^{-6} M; PC: 5.1×10^{-3} M; in 0.01 M pH 7 phosphate buffer, at 37 °C. Data are presented at the mean ± S.D. of three experiments. Lines represent a linear least-squares analysis.

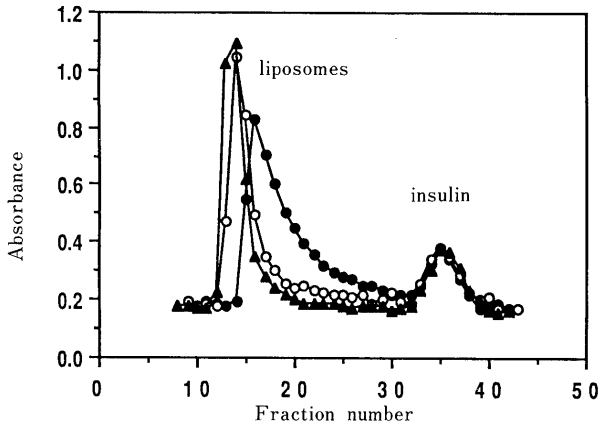

Fig. 3. Gel Filtration Profiles after Incubation of PC Liposomes with Insulin at 25 °C for 5 min

●, 30 nm extrusion; ○, 100 nm extrusion; ▲, 200 nm extrusion. Insulin: 1.7×10^{-5} M; trypsin: 4.3×10^{-6} M; lipid: 5.1×10^{-3} M; in 0.01 M pH 7 phosphate buffer.

Binding of Insulin to Various Sized Neutral Empty Liposomes Figure 3 shows the gel filtration profile after incubation of insulin and liposomes at 25 °C for 5 min. Insulin and liposomes were detected by Bio-Rad protein assay dye reagent. The 30 nm extruded liposome exhibited a tailing pattern in the liposome fraction, because the liposome size was small. We separated the liposome and insulin fractions according to this gel filtration profile. The amount of insulin bound to empty liposomes and lipid transferred to insulin was measured (Fig. 4). The amount of insulin bound to empty liposomes increased as the size of the empty liposomes decreased. The amount of lipid transfered to insulin was similar for all three liposome sizes.

Binding of Insulin to Various Electrically Charged Liposomes Figure 5 shows the gel filtration profile. The liposome fraction of the positively charged liposome con-

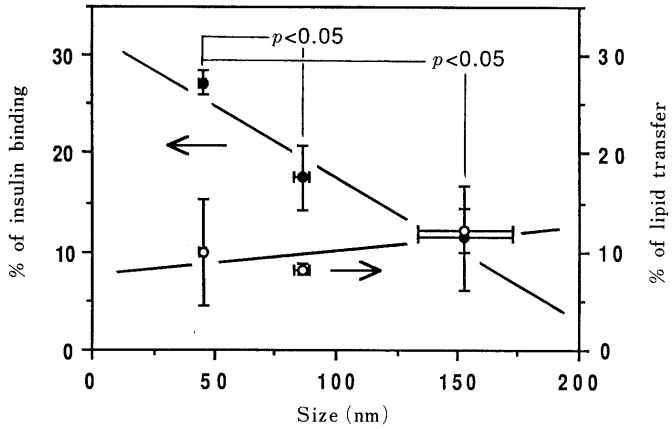

Fig. 4. Binding of Insulin to Liposomes and Lipid Transfer from Liposomes to Insulin after Incubation of PC Liposomes with Insulin at 25 °C for 5 min

●, insulin binding; ○, lipid transfer. Insulin: 1.7×10^{-5} M; trypsin: 4.3×10^{-6} M; lipid: 5.1×10^{-3} M; in 0.01 M pH 7 phosphate buffer. Data are presented as the mean ± S.D. of three experiments. Lines represent a linear least-squares analysis. Significant differences from free insulin according to Student's *t*-test.

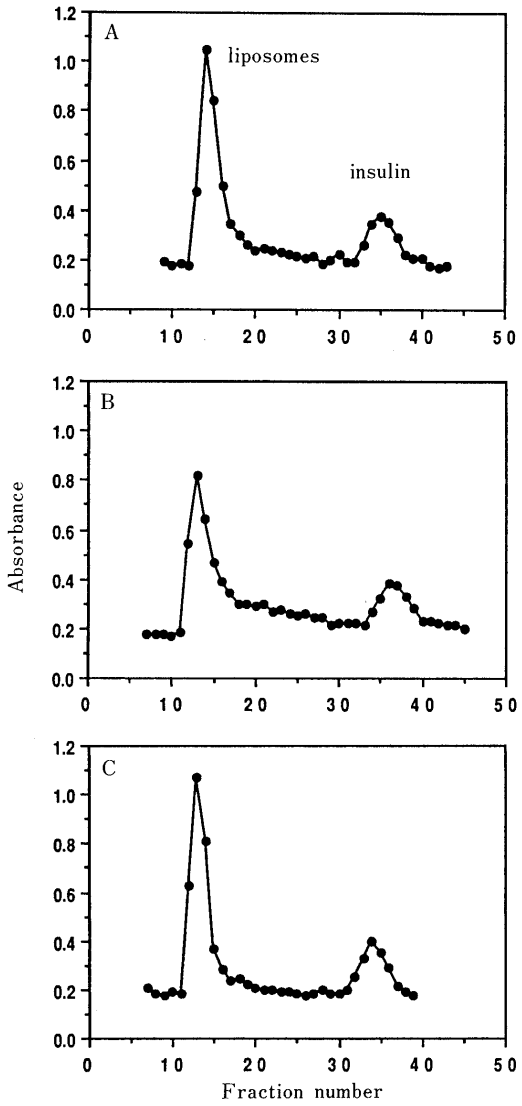

Fig. 5. Gel Filtration Profiles after Incubation of Various Charged Liposomes with Insulin at 25 °C for 5 min

A, PC liposome; B, StA/PC liposome; C, PA/PC liposome. Insulin: 1.7×10^{-5} M; trypsin; 4.3×10^{-6} M; lipid: 5.1×10^{-3} M; in 0.01 M pH 7 phosphate buffer.

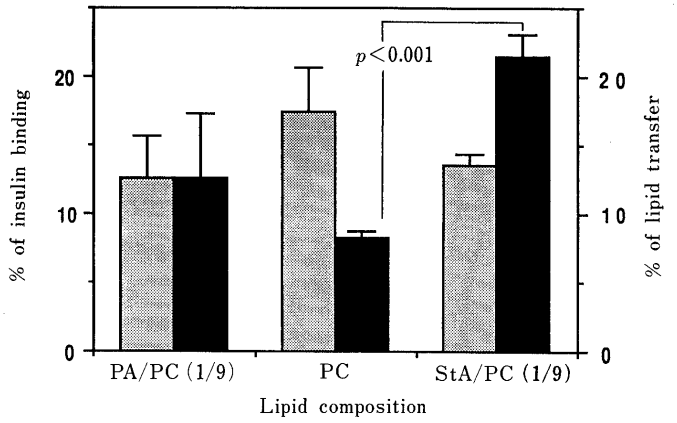

Fig. 6. Effects of Liposome Charge on Insulin Binding to Liposomes or Lipid Transfer from Liposomes to Insulin

▨, insulin binding; ■, lipid transfer. Insulin: 1.7×10^{-5} M; trypsin: 4.3×10^{-6} M; lipid: 5.1×10^{-3} M; in 0.01 M pH 7 phosphate buffer. Data are presented as the mean ± S.D. of three experiments. Significant differences from free insulin according to Student's *t*-test.

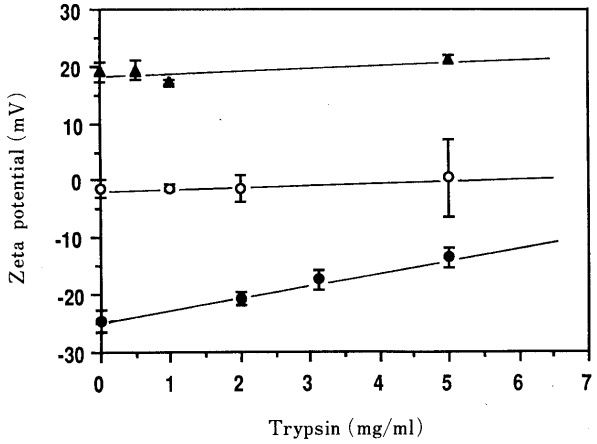

Fig. 7. Effects of Trypsin on Zeta Potential of Liposomes

○, PC liposome; ▲, StA/PC liposome; ●, PA/PC liposome. Lipid: 6.4×10^{-4} M; in 0.05 M pH 7 phosphate buffer. Data are presented as the mean ± S.D. of more than three experiments. Lines represent a linear least-squares analysis.

taining StA exhibited a tailing pattern. This tailing might have been caused by the electrostatic interaction between sepharose gel and the StA liposomes. The amount of liposome-bound insulin was greater for PC liposomes than for either StA/PC or PA/PC liposomes (Fig. 6). The amount of lipid transferred to insulin was as follows: StA/PC liposomes > PA/PC liposomes > PC liposomes (Fig. 6). There was a significant difference between StA/PC and PC liposomes.

Binding of Trypsin to Various Size Liposomes The extruded liposomes with sizes of 30, 100 and 200 nm did not show any zeta potential change after the addition of 0.5—5 mg/ml of trypsin (data not shown).

Binding of Trypsin to Various Electrically Charged Liposomes The 100 nm liposome was chosen to compare the binding of trypsin to the three electrical types of liposome using the zeta meter (Fig. 7). The zeta potential of negatively charged liposomes depended on the amount of trypsin added. The zeta potential of the negatively charged liposome was −24.5 mV without trypsin, and it increased to −13.5 mV after the addition of 5 mg/ml of trypsin. The zeta potential of neutral liposomes increased from

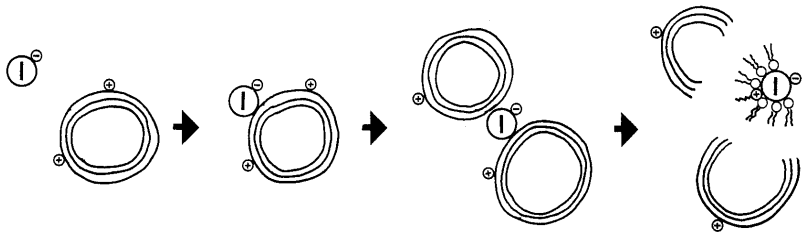

Fig. 8. Interaction between Insulin and Positively Charged Liposomes
Ⓘ, insulin.

−1.6 mV to +5.1 mV after the addition of 5 mg/ml of trypsin. The zeta potential of positively charged liposomes was unchanged by the addition of trypsin.

Discussion

Tryptic digestion of insulin was enhanced by reducing liposome size from 150 to 40 nm (Fig. 2). Previously, we suggested that an enzymatic reaction occurred on the liposome surface after the absorption of insulin and trypsin.[15] The tryptic digestion of insulin was enhanced by increasing the amount of neutral empty liposomes. The amount of insulin bound liposome increased as the liposome size decreased as measured by gel filtration (Fig. 4). Adsorption of proteins to the liposome was enhanced by reducing the liposome size.[24,27,28] This phenomenon has been attributed to the reduction in surface pressure with reducing liposome size.[28,29] On the other hand, the amount of lipid transfer from the liposome to insulin was estimated to be about 10%. This phenomenon may be similar to the lipid transfer from the liposome to high density lipoprotein (HDL).[30]

We found that the gel filtration technique was not suitable for studying trypsin binding to liposomes because the recovery of trypsin after gel filtration was less than 40%. Therefore, the zeta potential of the liposomes, with and without trypsin, was measured to investigate the phenomenon of trypsin adsorption to liposomes. This experiment was performed under different conditions (higher concentration of trypsin and lipid liposomes than in the study of tryptic digestion of insulin). The zeta potential was unchanged by the addition of trypsin to the 30, 100 and 200 nm extruded neutral liposomes. These experiments were performed in 0.05 M phosphate buffer in order to obtain reproducible data and this high ion concentration might have prevented trypsin binding to liposomes. Although the zeta potential of liposomes was examined, with and without insulin, no change in zeta potential could be observed after addition of insulin (the maximum insulin concentration could be used was 0.5 mg/ml because of low insulin solubility). The solubility of insulin may be a limiting factor for the detection of insulin binding to liposomes using the zeta meter.

Tryptic digestion of insulin was enhanced on the surface of liposomes after insulin binding.

The amount of lipid transferred from positively charged liposomes to insulin was higher than that from neutral and negatively charged liposomes. Figure 8 shows the proposed mechanism of the interaction between insulin and positively charged liposomes: insulin bound to positively charged liposomes and then the liposomes fused or aggregated. The lipid was transferred to insulin by electrical interaction

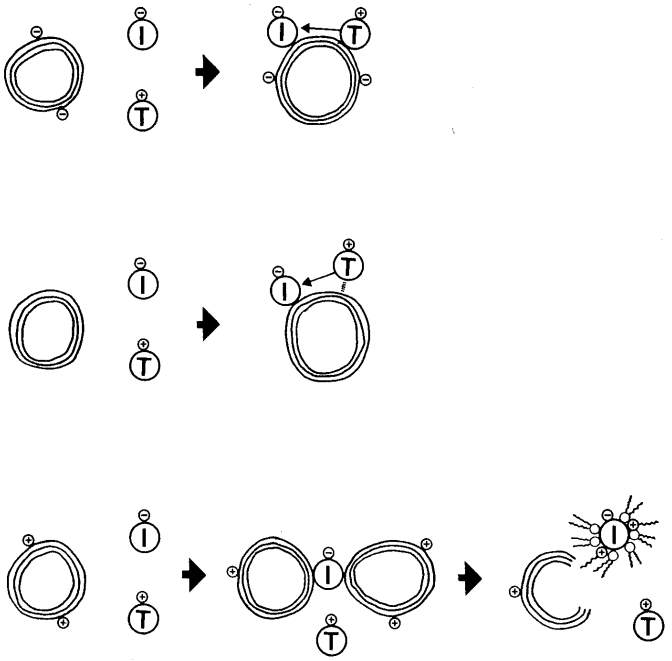

Fig. 9. Mechanism of Tryptic Digestion of Insulin Enhanced by Empty Liposomes
A, PA/PC liposome; B, PC liposome; C, StA/PC liposome. Ⓘ, insulin; Ⓣ, trypsin.

between the negatively charged insulin and the positively charged liposomes. Insulin may be protected from tryptic digestion by the lipid transfer proposed in Fig. 8. The mechanism protecting insulin from enzymatic digestion, by the addition of positively charged empty liposomes as reported by Weingarten *et al.*,[31] may be similar to that shown in Fig. 8.

There was no significant difference in the amount of liposome-bound insulin when neutral, negatively and positively charged liposomes were used. Large amounts of insulin can be easily bound to the liposome surface and insulin may be easily removed from this surface by various in the case of positively charged liposomes. More than 10% of the insulin was bound to be negatively charged liposome. These findings suggest that adsorption of insulin to the lipid membrane does not only occur due to an ionic interaction between insulin and lipid. Perry *et al.* reported that insulin was easily dissolved in PA/chloroform compared with PC/chloroform solution.[32] Positively charged amino acid residues such as Lys and Arg in the insulin molecule may interact with the negatively charged group in the PA molecule and negatively charged amino acid residues such as Glu and Asp in the insulin molecule may interact with the positively charged choline group in the PC molecule.

Trypsin binds strongly to negatively charged liposomes.

Trypsin is positively charged in solution at neutral pH, because the isoelectric point of trypsin is 10.1—10.8. Basic amino acids such as Arg and Lys bind to fatty emulsions containing egg PC which may include trace amounts of acidic phospholipids.[33] Basic proteins such as cytochrome c,[34] IFN-γ[35] and gramicidin S[36] have been reported to bind easily to acidic phospholipids. Recently, Liu and Huang reported that trypsin induced the release of the contents of liposomes which were composed of dioleoylphosphatidylethanolamine and oleic acid.[37] Trypsin may bind to negatively charged liposomes by the ionic interaction described, but binding of trypsin to neutral or positively charged liposomes was not observed. We previously reported that tryptic digestion of insulin was strongly enhanced by negatively charged liposomes and that neutral liposomes enhanced the digestion only weakly, while positively charged liposomes failed to enhance digestion.[15]

Figure 9 shows the mechanism of the enhancement of the tryptic digestion of insulin by empty liposomes that we propose. In the case of negatively charged empty liposomes, insulin and trypsin bind to the liposome surface, where trypsin easily attacks the insulin. In the case of neutral empty liposomes, insulin binds to the liposome surface and trypsin only weakly interacts with the liposomes. Then, tryptic digestion is enhanced compared with the situation without empty liposomes. In the case of positively charged liposomes, insulin binds to the liposome surface, then liposome fusion or aggregation and lipid transfer to insulin occurs immediately. The lipids surrounding insulin protect it from tryptic digestion.

In conclusion, gel filtration and the electrokinetic technique can help to elucidate the mechanism by which tryptic digestion of insulin is enhanced by empty liposomes. The present findings suggested that insulin leaking from the liposome may be digested by enzymes on the surface of the liposome. In the clinical administration of liposomal insulin, the physician should be aware of the possible interaction between insulin and liposomes. Our findings have been useful in investigating the opsonin mechanism of liposomes by serum proteins in the blood and also the mechanism of the enzymatic reactions that may take place on the surface of the membrane.

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