NOTE

No Reduction of Blood Glucose in Diabetic Rats after Oral Administration of Insulin Liposomes Prepared under Acidic Conditions

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Abstract

Insulin liposomes (dipalmitoyl phosphatidylcholine or dimyristoyl phosphatidylcholine/cholesterol=7/2) were prepared under strongly acidic conditions maintaining the biological activity of insulin. Although these were apparently noncharged liposomes, the association of insulin with membrane of liposomes was unexpectedly very large, and the uptake % of insulin was high. The distribution of vesicle-size of liposomes was narrow; the average diameter was $0.1\ \mu\text{m}$ and no multilayered structures were observed by electron microscopy. Oral administration of the insulin liposomes (15-30 IU/rat) in streptozotocin-diabetic rats did not result in any distinct tendency to reduce the level of blood glucose.

Some successful attempts were made, mostly by English pioneers to administer insulin orally using liposomes as a carrier (Dapergolas and Gregoriadis, 1976; Gregoriadis *et al.*, 1976; Patel and Ryman, 1976, 1977). Dapergolas and Gregoriadis (1977) succeeded in reducing blood glucose in streptozotocin-induced diabetic rats by oral administration of insulin liposomes. In their report, it is noteworthy that when dipalmitoyl or dimyristoyl phosphatidylcholine was used as a phospholipid constituent of the liposomes, a surprisingly small number of units of insulin could significantly suppress the level of blood glucose. Recently we reported that positively charged insulin liposomes were considerably stable when incubated with proteolytic enzymes, and that they reduced blood sugar and increased glucose tolerance by oral administration in half the alloxan-diabetic rats tested (Hashimoto and Kawada, 1979). The present

paper reports the failure of gastric administration of insulin liposomes constituted of dipalmitoyl or dimyristoyl phosphatidylcholine and cholesterol to decrease blood glucose in streptozotocin-diabetic rats.

Materials and Methods

Preparation of insulin liposomes

Dipalmitoyl phosphatidylcholine (DPPC) or dimyristoyl phosphatidylcholine (DMPC), both from Sigma Chem. Co., and cholesterol (Wako Pure Chem. Indust., Tokyo) were dissolved in chloroform at a molar ratio of 7:2 (Total amount of lipids was about 30 mg). A thin layer of the lipids was formed on the inside wall of a flask by evaporating the solvent at 55°C under vacuum. Insulin (25.3 IU/mg), a gift from Dr. R. E. Chance of the Lilly Research Laboratories, was dissolved in 0.01 N HCl (40 IU/ml). Two ml of the solution was placed in the flask and indirectly sonicated for 1 min at 45°C using a cup-horn. The resulting suspension was kept for 60 min at the same temperature, then sonicated again for total 2 min with cooling at 4°C , using a 12 mm titanium probe at 150 W in a Branson Sonifier, Model W-185. The suspension was kept for 60 min at 4°C . The fraction of liposomes was ob-

tained by Sephadex G-75 column chromatography using 0.01 N HCl as an eluant. When isotonic liposomes were prepared, all HCl solution used was made isotonic with glycerine.

Preparation of ^{125}I -insulin

To determine the rate of association of insulin with liposomes, ^{125}I -labeled insulin was used as a marker. The labeling was done according to the method of Wajchenberg *et al.* (1978) with slight modification; the reaction was carried out at pH 2.0 and at the separation of ^{125}I -insulin by Sephadex G-75 column chromatography, 0.01 N HCl solution was used as an eluant.

Electron microscopy of liposomes

The shape and size-distribution of DPPC-containing insulin liposomes were observed in an Hitachi AU-12 electron microscope after negative staining with 1% uranyl acetate.

Animal experiments

Male Sprague-Dawley or Wistar rats were used. Streptozotocin (STZ) (Sigma) was *iv* administered (60 mg/kg body wt.) (Junod *et al.*, 1969) when the rats were 5 to 6 weeks old and insulin liposomes (2 ml/rat, the amount of total lipids was 13–15 mg/rat) were given by stomach tube 5 or 6 days after the STZ injection. Rats always had free access to diets during the experimental period (Dapergolas and Gregoriadis, 1976, 1977).

Determination of blood glucose

The conventional method for measuring glucose with o-toluidine-boric acid (OTB) reagent was modified to facilitate microdetermination. Fifty μl of a blood sample obtained from the tail vein was deproteinized with 4 ml of 0.33 M HClO_4 . After centrifugation, 0.3 ml of the supernatant was mixed with 3.0 ml of OTB-reagent. In this method, good linearity was observed in a glucose concentration of 0 to 800 mg/dl in the standard curve.

Results and Discussion

Insulin liposomes

The rate of association of insulin with liposomes thus prepared was very high; for DPPC-liposomes about 75% and for DMPC-liposomes nearly 35%, respectively. These high values indicate that interaction between insulin and the membrane of the liposomes is of a distinct nature. This is

probably due to the influence of the strongly acidic pH used in the preparation, since less than 10% association was found when insulin liposomes were prepared at pH 7.5. When liposomal lipids were analyzed by thin layer chromatography after extraction of lipids with a chloroform-methanol (2:1) mixture, a slight trace of free palmitic acid was detected on the DPPC-liposomes prepared at pH 2.0 and none on those at pH 7.5. However, a relatively high cholesterol content would satisfactorily maintain the function of the liposomes. At present, we cannot explain what happens in the mechanism of association between insulin and neutral lipid membranes under the condition of acidic pH. As shown in the electron microscopy photograph (Fig. 1), the size of the liposomal particles was fairly uniform; the average diameter was 0.1 μm and no multilayered structure was observed, although irregularly shaped and multilamellar liposomes were found at the time of the first sonication. When these liposomes were kept at 4°C, no noticeable morphological change was detected even after 2 weeks of storage.

Oral administration of insulin liposomes

Before the administration of liposomes, the circadian variation of the level of blood glucose was examined in the STZ-diabetic rats. As no tendency to a decrease in blood glucose was demonstrated between 19:00 to 04:00 in our experimental conditions (Fig. 2), all of the animal experiments in which liposomes were administered were carried out within this period of time. The effect of insulin liposomes intragastrically administered on blood glucose is summarized in Table 1. However, IRI was increased, 1 hr after the administration of insulin liposomes (26 IU/rat), from 68 $\mu\text{U}/\text{ml}$ to 134 $\mu\text{U}/\text{ml}$ in the serum and from 13 $\mu\text{U}/\text{ml}$ to 40 $\mu\text{U}/\text{ml}$ in the thoracic lymph respectively. When the insulin liposomes were directly given into the jejunum, IRI

Fig. 1. Insulin liposomes of 7:2, dipalmitoyl phosphatidylcholine: cholesterol prepared at pH 2.0, negatively stained with uranyl acetate. Magnification $\times 50,000$; bar is $0.1 \mu\text{m}$.

in the serum was increased 25 times.

These findings show that the administered insulin was effectively absorbed from the jejunum into the blood stream. In contrast to this effective absorption, serum insulin was doubled only when an insulin solution of pH 2.0 (29 IU/rat) was administered into the jejunum. In spite of the increase in serum IRI, no definite changes in blood glucose were observed over 4 hr after administration, irrespective of the differences in the phospholipid composition of liposomes, isotonicity, and the strain of rats. Dapergolas and Gregoriadis (1977) showed that a nearly 55% reduction in blood glucose was attained by oral administration of the same composition of DPPC-liposomes (2.9 IU) or DMPC-liposomes (0.3 IU) at 4 hr. In the latter case, it was long-acting and was still significant at 24 hr. The only difference between their experiments and ours can probably be ascribed to the method used in preparing the liposomes. We prepared them in an acidic medium which was the stable conditions for insulin. As mentioned above, our liposomes had no multilayered structure despite the short-term

Fig. 2. Variation in the levels of blood glucose of streptozotocin-induced (50 mg/kg body wt.) diabetic Wistar rats. Observation was made at 5th day of streptozotocin. Animals had free access to diets. Values are Mean $\pm$ SE ($n=12$).

Table 1. Effect of oral administration of insulin liposomes on blood-glucose level of STZ-diabetic rats.

	No. of rats	Blood-glucose level (% of initial)	
		2 h	4 h
Sprague-Dawley rats:			
Control ¹⁾	10	103 ±4	96.0±4.2
DPPC-liposomes ²⁾	9	88.4±3.5 (p<0.02)	96.5±5.6 (NS)
DMPC-liposomes ³⁾	12	114 ±6 (NS)	110 ±5 (NS)
Control ⁴⁾	13	98.9±4.1	94.5±5.0
DPPC-liposomes ⁵⁾ (isotonic)	13	97.8±5.3 (NS)	97.2±5.6 (NS)
Wistar rats:			
Control ⁶⁾	8	87.1±5.9	81.5±4.9
DPPC-liposomes ⁷⁾	10	87.0±4.2 (NS)	93.5±3.2 (NS)
Control ⁸⁾	9	89.6±2.9	86.7±3.7
DPPC-liposomes ⁹⁾ (isotonic)	9	108 ±4 (<0.05)	97.7±4.4 (NS)

Initial blood-glucose level (Mean ± SE, mg/dl) and units of insulin/rat are as follows; 1) 420 ± 17, free Insulin 30 IU, 2) 348 ± 23, 30 IU, 3) 344 ± 22, 15 IU, 4) 476 ± 28, isotonic glycerine solution (pH 2.0), 5) 490 ± 19, 30 IU, 6) 448 ± 24, free insulin 20 IU, 7) 400 ± 20, 25 IU, 8) 457 ± 23, isotonic glycerine solution (pH 2.0), and 9) 356 ± 32, 30 IU.

sonication during preparation. In addition the apparent uptake % of insulin was unusually high, indicating a strong interaction of the protein with liposomal membranes (Tyrrell *et al.*, 1976). This is probably the specific nature of liposomes prepared under acidic conditions. However, we cannot explain why such liposomes were ineffective when administered orally. The critical differences between the characteristics of effective and noneffective liposomes remain to be clarified.

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