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COVALENT ATTACHMENT OF INSULIN TO THE OUTER SURFACE OF LIPOSOMES

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We describe a method for covalent binding of insulin to the outer surface of multilamellar liposomes loaded with spin label. Encapsulation of the label Tempocholine-nitroxide within the aqueous phases of liposomes is controled by Electron Spin Resonance. The binding of insulin is performed using the Carlsson's heterobifunctional reagent : N-succinimidyl 3-(2-pyridyldithio) propionate. The coupling method results in efficient attachment of 2. 64.10^{-4} mole of insulin per mole of phospholipid ; the integrity of these vesicles is not modified as confirmed by spin resonance analysis. Moreover, the liposome-coupled insulin retains its antigenic specificity as shown by radioimmunoassays.

A number of recent studies have focused on the use of macromolecular ligands as a means of promoting specific liposome-cell binding "in vitro" (1). For liposome targeting, antibodies molecules were covalently coupled to phospholipids (2), fatty acids (3, 4, 5) or to preformed liposomes (6,7,8). Several authors used the Carlsson's heterobifunctional reagent : N-succinimidyl 3-(2-pyridyldithio)propionate, SPDP (9 to 12) for such a purpose.

In the present study, we describe the covalent attachment of insulin to the surface of multilamellar liposomes by SPDP reaction. Such sensibilized vesicles by an outer surface peptidic antigen are of interest in immunochemistry of membranes during antibody - antigen interactions, and for promoting the production of natural antibodies by increasing the biodisponibility of the peptide molecule.

Moreover, these vesicles loaded with an hydrosoluble spin label could permit the development of a new method for biochemical analysis of hormonal polypeptides (13) based on Electron Spin Resonance (ESR) spectroscopy, after their induced immuno-lysis by addition of anti-insulin antibody and complement.

MATERIALS AND METHODS

Materials - Egg yolk lecithin (L-α phosphatidylcholine) (PC), dipalmitoylphosphatidylethanolamine (PEA), cholesterol (CHOL), dicetylphosphate

0006-291X/83 $1.50

(DCP) and bovine insulin were purchased from Sigma. SPDP, Sephadex G 15, G 25 and Sepharose 4 B were obtained from Pharmacia. Bio-Sil Ha was from Bio-rad. Dithiothreitol (DTT) was from Ega C. Silicagel 80 TCL (sheet plates) were from Merck (Ref : 5626).

All ESR spectra were recorded with a Brucker ER 200 D X band spectrometer, equipped with a double cavity (ER 4105 DR) mode TE 104.

Liposomes and proteins were concentrated on an Amicon ultrafiltration cell equipped with YM 10 and YM 5 filters respectively.

Insulin radioimmunoassay was done using the reagents of the INSIK 5 CEA(ORIS)/SORIN (Saclay, France) Kit.

Methods - Protein concentrations were determined by the method of Lowry and al. (14) with bovine insulin as standard and by radioimmunoassay. Lipid phosphorus contents were estimated by the method of FISKE and SUBBAROW (15).

The spin label Tempocholine was prepared as described by KORNBERG and Mac CONNELL (16).

All the different steps of the following procedure are summarized in figure 1.

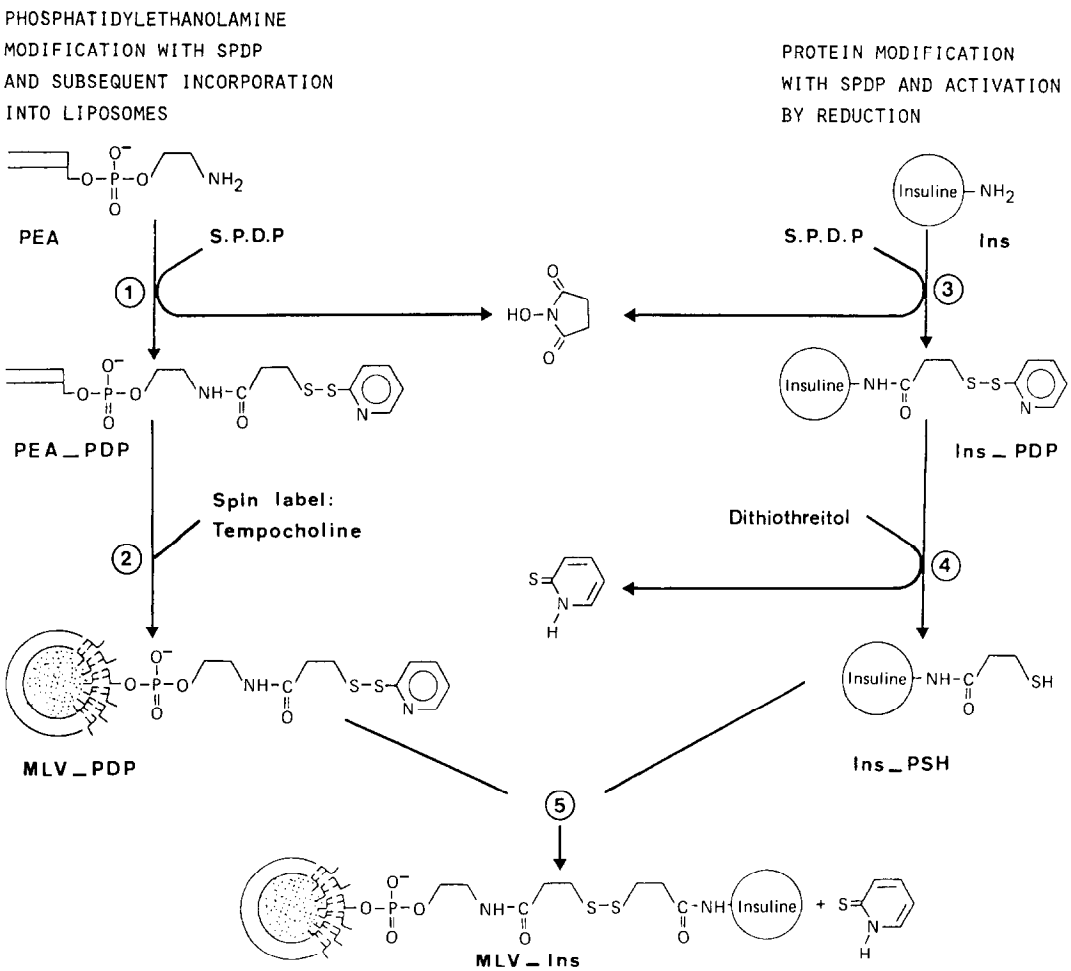

Figure 1 - Covalent coupling of insulin to spin labeled liposomes

Synthesis of N-(3-(2-pyridyldithio)propionyl)phosphatidylethanolamine (PEA-PDP) (step ①):

PEA-PDP was synthetized by using the method of MARTIN and al (11). PEA (20 µmoles) was dissolved in 1.2 ml of anhydrous solution of chloroform/ Methanol 9:1 containing 20 µmoles of triethylamine and 32 µmoles of SPDP.

The reaction was carried out at 25°C under nitrogen during 5.5 hours. The mixture was applied to a column poured with silicagel activated overnight at 150°C. The column was washed with an additional 20 ml of each of the following chloroform/methanol mixtures (20/0 ; 20/0.5 ; 20/0.8 ; 20/1.0 ; 20/1.5 and 20/2.0). The phosphate containing fractions eluting in 20/2 chloroform/ methanol were concentrated under reduce pressure.

Preparation of spin-labeled and PDP-sensitized liposomes (step ②):

Multilamellar vesicles (MLV) were prepared by the method of KINSKY and al (17). 8 µ moles of PC, 8 µ moles of CHOL, 1.76 µ moles of DCP and 0.04 µ moles of PEA-PDP were mixed and the organic solvent was evaporated under nitrogen. To the lipid film was added 0.8 ml buffer (borate buffer 0.1 M, 0.1 M NaCl, pH 7.5) containing 0.065 M Tempocholine. Lipids were suspended by vortexing and liposomes were allowed to swell two hours at room temperature. Unencapsulated Tempocholine was removed by gel filtration through a Sephadex G 15 column, and the eluated liposomes fractions concentrated on an Amicon YM 10 membrane filtration.

Introduction of 3-(2-pyridyldithio)propionyl group (PDP) into insulin (steps ③ and ④):

The PDP groups were introduced into insulin by reaction with SPDP as described by CARLSSON and al (18). Prior to SPDP modification, insulin (dissolved with methanol/phosphate buffer 0.1 M, 0.1 M NaCl, pH 7.5 at a ratio of 50/50) was transferred to phosphate buffer 0.1 M, 0.1 M NaCl, pH 7.5 by gel filtration through a Sephadex G 25 column. Insulin was incubated with a SPDP solution (40mM in methanol) for 30 min.at 23°C (molar ratio SPDP/insulin (20/1)). The SPDP reagent was removed by filtration through a Sephadex G 25 column and the insulin transferred to acetate buffer 0.1 M, 0. 1 N NaCl, pH 4.5. This insulin modified solution was filtered on an Amicon YM 5 membrane.The PDP to protein ratio was determined as follow : PDP-insulin was treated with dithiothreitol (final concentration of 25 mM) at room temperature. This treatment entails the release of pyridine 2-thione, which has a molar absorptivity of 8.08×10^3 at 343 nm (19). The amount of pyridine 2-thione released is equivalent to the content of PDP groups in PDP-insulin. DTT in excess was separated through a Sephadex G 25 Column (borate buffer 0.1 M, 0.1 M NaCl, pH 7.5), and the eluated modified insulin fractions concentrated once more on an Amicon YM 5 filter.

- Coupling of PSH - Insulin to liposomes containing PEA-PDP (Step ⑤): PSH - insulin was added to liposomes containing PEA-PDP (0.5 ml of insulin solution 0.13 mg/ml with 0.5 ml of liposomes preparation 1.87×10^{-3} M in phospholipids (PL)) and incubated at room temperature for 20 hours. Protein bearing liposomes were separated from uncoupled protein by gel filtration through a Sepharose 4B column equilibrated in borate buffer 0.1 M, 0.1 M NaCl, pH 7.5 and concentrated by filtration on an Amicon YM 10 membrane.

- ESR measurements :

Samples were contained in 50µ l capillar Corning Glass corp. Parameters of analysis were : microwave power 10 mW ; scan time 200 s ; time constant 200 ms and modulation amplitude 2 G.

- Radioimmunoassay (RIA):

The binding of insulin with liposomes was measured after dilution in saline phosphate buffer, pH 7.2, BSA 0,1 %, by radioimmunoassay using polyethylene glycol as precipitation adjuvant in the double antibody technique.

RESULTS AND DISCUSSION

The phosphate - containing fractions eluting in 20 : 2 chloroform/methanol after reaction ① was identified as PEA-PDP as follow : thin layer chromatography with chloroform/methanol/acetic acid 30 : 10 : 1.5 indicated a single spot with Rf : 0.75 ; this product was ninhydrine negative, iodine and rhodamine positive ; the yield was 75 %, based on lipid phosphorus assay. PEA-PDP was stored under nitrogen at -15°C.

The obtention of liposomes (step ②) was determined by phosphorus assay and their integrity was revealed by ESR measurements. We observed a quenching of the nitroxide spin probe due to its high concentration into the aqueous phases of the liposomes, and high dipolar interactions between free electrons. The content of this label into vesicles was determined, after lysis by Triton X 100, by the intensity of the isotropic triplet signal due to the dilution of the probe into the buffer. In these preparations, 400 nmoles of nitroxides were encapsulated per 1 μmole of phospholipid ; liposomes are stable during several weeks despite a small release of the marker during the first days according to our previous works (20).

For the molecular sensibilisation of insulin and its attachment to the outer surface of liposomes (steps ③ to ⑤) the results are summarized in table 1.

Figure 2 (curve A) shows the elution pattern obtained after chromatography through a Sepharose 4 B column of MLV-PDP incubated with insulin-PSH. The presence of MLV and insulin was determined by the absorbance of the effluent at 280 nm. The eluated fractions corresponding to MLV was passed through the same column (curve B). We haven't observed any release of insulin during this second purification ; this is a confirmation of the covalent attachment of insulin to liposomes.

After the first purification and concentration on YM 10 filter (step ⑤), we have measured, on an aliquot of the MLV fraction, the quantity of insulin fixed to the outer surface of these liposomes by radioimmunoassay. We noted that $2.64.10^{-4}$ mole of insulin were fixed per mole of phospholipid and the absolute concentration of immunoreactive insulin estimated at 0.32 μg/ml for the prepared samples. Owing to the fact that the multilamellar vesicles contains phospholipids on all the bilayers, these values show a good ratio between antigen covalently bound to the surface and phospholipids head groups on the outer sphere of liposomes (up to 10^{-3}). We haven't noted significant variations of the immunoreactive-insulin/insulin-protein (measured by lowry method) ratio during the various intermediate steps of insulin modification. Attempts to determine the protein quantity by Lowry method in the last sample (MLV-Ins) were unsuccessful, according to the very low concentration of insulin fixed to vesicles.

Table I - Component concentrations during covalent attachment of insulin to multilamellar liposomes

Steps (††)	Compounds		Insulin (*) mg/ml	Insulin (*) yield %	Liposomes PL(M)	Tempocholine (M)	Tempocholine nmole/mole PL
	Commercial insulin		5.50				
	Purified Insulin	(a)	0.70±0.10	80.3			
		(b)	5.00±0.60	74.5			
③	Ins-PDP	(a)	0.11±0.04	21.2			
		(b)	0.60±0.14	61.0			
④	Ins-PSH (**)	(a)	0.03±0.01	34.9			
		(b)	0.11±0.02	69.0			
②	MLV-PDP	(a)	-	-	0.67×10^{-3}	2.7×10^{-4}	402
		(c)	-	-	1.87×10^{-3}	7.7×10^{-4}	411
⑤	MLV-Ins	(c)	0.32×10^{-3} (♯)	-	2.00×10^{-4}	4.0×10^{-5}	200

PL : phospholipid
(a): after chromatography through Sephadex G 25
(b): after concentration on Amicon YM 5 membrane
(c): after concentration on Amicon YM 10 membrane
(*): concentration measured by Lowry method (on 4 different experiments).
(**):pyridine 2-thione assay revealed that 0.3 mole of PDP were fixed per 1 mole of insulin
(♯): concentration measured by radioimmunoassay
(††): steps corresponding to fig. 1

Nevertheless, the radioimmunoassays confirm the efficient binding of insulin to the outer surface of multilamellar liposomes with preservation of its antigenic activity. In the other hand,we have incubated multilamellar liposomes with insulin, without any covalent attachment. After purification of these vesicles, any adsorption of insulin to the outer surface of these MLV was detected by RIA, according to similar results for MLV by WIESSNER and HWANG (21).

Concerning the stability of our insulin sensitized liposomes, we have not observed any alteration of the vesicles fifhteen days after preparation, on storage at + 4°C. After this delay, we observed a quenched ESR Tempocholine signal corresponding to 200 nmoles of nitroxide encapsulated per mole of phospholipid (see figure 3a). Chemical lysis by Triton X100 resulted in a high enhancement of the ESR signal, which is in agreement with a good integrity of the vesicles prior to lysis (figure 3b).

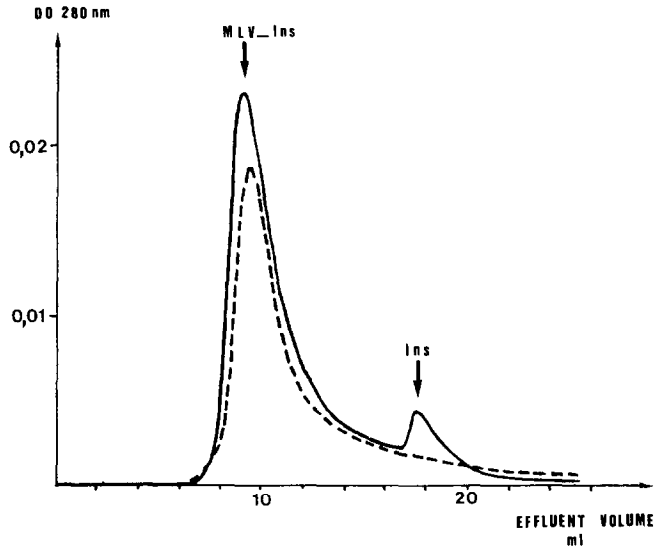

Figure 2 - Sepharose 4B elution profile of insulin bound to liposomes :
Curve A : Purication of MLV - insulin (——)
Curve B : Control of non insulin release form MLV - insulin (----)

CONCLUSION During this set of experiments, we have shown that : multilamellar vesicles loaded with Tempocholine conserve their integrity when insulin is covalently attached to the surface ; chemical lysis results in spin label release with enhancement of its ESR signal. Despite the fact

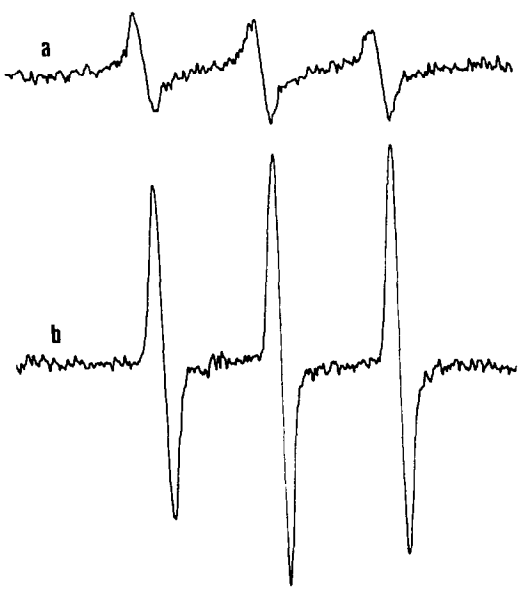

Figure 3 - ESR spectra of MLV-insulin loaded with Tempocholine (gain : 5.10^5). Insulin is covalently coupled to the outer surface of these liposomes
-a- before lysis
-b- after chemical lysis

that it was not possible to quantify by the Lowry method the presence of insulin on lipids layers, we have shown by RIA that binding of insulin to the outer surface of liposomes is efficient at a level of $2.64.10^{-4}$ mole of insulin per mole of phospholipid. Radioimmunoassays show essentially the preservation of immunoreactivity of the peptidic hormone coupled by a disulfure bond to the polar head of phospholipid. These results show also that these peptidic-sensitized and spin-labeled vesicles are potentialy interesting labels for cell biology applications.

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