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Preparation of superoxide dismutase entrapped in ceramide-containing liposomes for oral administration

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Abstract

Ceramides and distearoylphosphatidylcholine (DSPC) were incorporated in various liposomes preparations in comparison with dipalmitoylphosphatidylcholine alone. Preparations were introduced in an 'Artificial Stomach-Duodenum' model to improve their stability. Better results were observed for DSPC and ceramide-containing liposomes. Entrapment of Cu-Zn Superoxide dismutase (SOD) in liposomes have been carried out for oral administration. The efficiency of entrapment of SOD was 35.4% for liposomes without ceramides, and from 24.3% to 46.1% for ceramide-containing liposomes.

Keywords: Ceramides; Liposomes; Multilamellar vesicles; Distearoylphosphatidylcholine; Superoxide dismutase

Ceramides, a combination of sphingosine and amide-linked fatty acid, demonstrate various biological effects such as membrane receptors for bacteria, viruses, immunoregulatory molecules, and are believed to be involved in cell-cell recognition phenomena. They constitute an important element in the construction of the lipid bilayer, and are thought to maintain the multi-bilayer organization of barrier lipids. However, they are able to forme multilamellar vesicles in the absence of phospholipids. This lamellar forming ability is suggested to be involved in their water retaining properties.

There is a growing of interest in ceramides as elements of liposome formulation. Entrapment of biologically active substances in liposomes is an efficient method for delivery of macromolecules to isolated cells in vitro or target organs in vivo. Liposomes are now recognized as a drug delivery system that can improve the therapeutic activity

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and safety of a wide range of compounds. Superoxide Dismutase (SOD), an enzyme that scavenges superoxide anion, is one of these products which should benefit of a such specialized drug delivery system (Michelson, 1986; Freeman et al., 1983).

Distearoylphostidylcholine (DSPC), and dipalmitoylphosphatidylcholine (DPPC) was obtained from D3F (Paris, France). Cholesterol (CHO), stearylamine and sodium chloride in 0.9% solution were supplied by Pharmacie Centrale des Hôpitaux (Paris, France). Ceramides (CER) were extracted and purified from wheat by INOCOSM (Châtenay Malabry, France). Bovine erythrocyte Cu-Zn SOD (3685 U/mg) was obtained from Allerbio (Varennes-en-Argonne, France).

Liposomes entrapping SOD were prepared by a modification of a patented injection method (no. 89401 857-1). Briefly, stearylamine was dissolved at 40°C in 2 ml of chloroform. After evaporation of the chloroform, DSPC or DPPC and cholesterol were added to stearylamine, and dispersed in 20 ml of ethanol. For preparations including ceramides, addition to ethanolic solution has been carried out after dissolution at 80°C. The aqueous phase was prepared by addition of 2 mg of SOD in 40 ml of 0.9% NaCl solution. The lipid phase placed in a syringe was then admixed to the aqueous phase at 25°C, using an UltraTurrax (IKA, Stauffen, Germany). Resulting liposomes were then dried on a rotary evaporator at 65°C under reduced pressure to eliminate ethanolic phase.

In order to determine their stability for oral administration, 'empty' liposomes were tested in an "Artificial Stomach-Duodenum" model previously described (Vatier et al., 1994). This model simultaneously simulates gastric secretory flux, the gastric emptying rate, and bicarbonate secretory fluxes corresponding to duodenal alkaline secretion and to alkaline pancreatic secretion. Turbidity increase as probe of structural degradation was evaluated at 400 nm in stomach and duodenum compartment, according to the method of Nagata (Nagata et al., 1988). Variations of molar concentrations after 2 h, were 0.8 mM for DPPC-without ceramide-containing liposomes, were from 0.25 to 0.35 mM for DPPC and ceramide-containing liposomes, and were 0.06 mM for DSPC- and ceramide-containing liposomes. These data demonstrated that DSPC was more resistant than DPPC to gastric attack, and that ceramides potentiated this effect. Thereafter, ceramides and DSPC were retained as preferable liposome constituents, to protect superoxide dismutase from gastric attack in the course of oral administration. Various preparations were performed to optimize enzyme entrapment.

An electron microscopy study has been carried out. Formvar-coated grids stabilized with carbon were made hydrophilic by glow discharge and floated on the liposome suspensions for 1 min. After rinsing with 3 exchanges of Tris, pH 7.4 (10 mM Tris, 10 mM NaN_3, 1 mM $MgSO_4$), the preparations were negatively stained with 1.5% uranyl acetate in distilled water. All preparations showed exclusively multilamellar vesicles (MLV) (Fig. 1). These liposome suspensions were stable and did not sediment, even after 1 month of incubation at 37°C.

Liposome size was determined by a nanosizer (Coultronics), and confirmed by electron microscopy. SOD entrapment was evaluated by SOD activity measurement at 550 nm, according to the technique of McCord and Fridovich (McCord and Fridovich, 1969), after appropriate dilution of liposomes in a PBS + 1% Triton X-100 solution (Table 1). The efficiency of SOD entrapment was 35.4% for liposomes without ceramides, and from 24.3% to 46.1% for ceramides-containing liposomes. As shown in Table 1, maximal entrapment of SOD was obtained with ceramide-containing liposomes with a molar ratio of (14/7/4/1). Incorporation of ceramides into preparations increased the size of liposomes.

These results demonstrated that DSPC and ceramides enhanced resistance of liposome membranes to enzyme attack, and that in a molar ratio of (14/7/4/1), ceramide enhanced SOD entrapment. This composition was found to be suitable for further in vivo studies (Regnault et al., 1995, 1996).

Fig. 1. Negative staining of ceramide-containing liposomes. The liposomes are multilamellar. × 50 000, (bar, 100 nm).

Table 1
Comparison of size and entrapment of SOD in different liposomes ($n = 8$).

Composition (molar ratio)	Size ± S.E.M. (nm)	Dispersion	Entrapment of SOD (%)
DSPC/CHO/Stearylamine (14/7/4)	255 ± 25	2	35.4
DSPC/CHO/Stearylamine/CER (14/7/4/14)	674 ± 181	6	24.3
DSPC/CHO/Stearylamine/CER (14/7/4/7)	556 ± 170	5	32.7
DSPC/CHO/Stearylamine/CER (14/7/4/4)	525 ± 143	4	36.8
DSPC/CHO/Stearylamine/CER (14/7/4/1)	487 ± 114	4	46.1

Entrapment of SOD is expressed as % of SOD in liposomes after 7 days of storage at 4° C.

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