Development of a Novel Bioactive Formulation of Vasoactive Intestinal Peptide in Sterically Stabilized Liposomes

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INTRODUCTION

Vasoactive intestinal peptide (VIP), a 28-amino acid neuropeptide, displays a broad range of biological activities (1). Widely distributed in the central and peripheral nervous system, it has been shown to induce vasodilation in several vascular beds leading to a decrease in mean arterial pressure (2–5). However, as many peptides, the hypotensive effect of VIP when administered intravenously as an aqueous solution is relatively short-lived. Gao et al (2) and Suzuki *et al.* (3–4) have recently established that encapsulation of VIP into conventional liposomes decreased systemic arterial pressure after i.v. injection in anesthetized hamsters, potentiated and prolonged arteriolar vasodilation *in situ*. This improved activity of liposomal VIP can be explained by several mechanisms including protection from enzymatic degradation (2,5). Furthermore, some studies have described VIP molecules in a random coil conformation in aqueous solutions, whereas organic solvents and anionic lipids induced the formation of a π-helix that has been correlated to an increase in activity (6,7). Since VIP has the ability to bind and penetrate lipid bilayers (8), a liposomal formulation may thus enhance the biological response of the peptide, by providing an optimal hydrophobic environment for the formation of its active helical conformation.

Unfortunately, the therapeutic parenteral use of conventional liposomes is limited by their rapid uptake by the reticuloendothelial system, issue that has been recently overcome by the development of sterically stabilized liposomes (SSL) as an improved drug delivery system (9). SSL are polymer-coated liposomes, where the polymer, in most cases polyethylene glycol (PEG), is covalently conjugated to one of the phospholipids and provides a hydrophilic cloud outside the vesicle bilayer. This steric barrier delays the recognition by opsonins, and as a result, SSL remain in the blood much longer than conventional liposomes (9–12). An increase in the pharmacological efficacy of encapsulated agents in SSL could hence be anticipated, and was demonstrated for some chemotherapeutic and anti-infectious drugs (9).

The purpose of this study was therefore to attempt to apply the recently developed SSL technology to the intravenous administration of VIP, expecting to prolong the overall efficacy of the peptide compared to its delivery in aqueous media.

MATERIALS AND METHODS

Chemicals

Egg yolk phosphatidylcholine (PC), egg yolk phosphatidylglycerol (PG), cholesterol (Chol) and trehalose were purchased from Sigma Chemical Co. (St. Louis, MO). Polyethylene glycol (PEG) covalently linked to distearoyl-phophatidylethanolamine ($PEG_{1,900}$-DSPE) was kindly provided by Sequus Pharmaceuticals (Menlo Park, CA). VIP was purchased from American Peptide Co. (Sunnyvale, CA).

Liposomes Preparation Methods

Two different preparation methods were successively used. In both cases initially, the lipids (PEG-DSPE:PC:PG:Chol, molar ratio 0.5:5:1:3.5, total phospholipid content 17 μmol) were mixed in chloroform in a round bottom flask. The solvent was evaporated at 45°C in a rotary evaporator (Labconco, Kansas City, MO) and dessicated under vacuum overnight.

Method A: Freeze-Thaw/Extrusion

The dry lipid film was rehydrated with 250 μl 0.15 N saline containing 0.4 mg VIP. The mixture was vortexed, sonicated for 5 minutes in a 175.5W water bath sonicator (Fisher Scientific, Itasca, Il), and freeze-thawed five times in a acetone-dry ice bath. The suspension was extruded through polycarbonate filters using the Liposofast apparatus (pore size 200 nm, AVESTIN, Inc., Ottawa, ON, Canada). The liposome-associated VIP was separated from the free VIP by column chromatography (Bio-Gel A-5m, Bio-Rad Laboratories, Richmond, CA) and stored at 4°C for maximum 15 days.

Method B: Extrusion/Dehydration-Rehydration

The dry lipid film prepared as before was rehydrated with 250 μl 0.15 N saline only. The solution was vortexed, bath-sonicated for 5 minutes, and extruded through stacked polycarbonate filters to give a vesicle size of about 80 nm (pore size 200, 100, 50 nm). VIP (0.4 mg) and trehalose (30 mg) as a cryoprotectant were added as powder forms to the extruded suspension. The mixture was then frozen in an acetone-dry ice bath and lyophilized at −46°C under a pressure of $\sim 5.10^{-3}$ M Bar overnight (Labconco "Freezone 6", Kansas City, MO). The lyophilized "cake" was resuspended with 250 μl deionized water. Column separation and storage conditions were the same as above. The SSL prepared by A had a final mean size of 224 ± 36 nm and the SSL prepared by B an average diameter of ~250 ± 50 nm. For both, the recoveries were ~30% for VIP and ~50% for phospholipids, giving a ratio of ~0.004 mole VIP/mole of phospholipid.

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In Vitro Characterization of VIP in SSL

Size Determination

The size of the vesicles was determined by quasi elastic light scattering (15) with a Nicomp 270 particle sizer (Particle Sizing Systems, Santa Barbara, CA).

Phospholipid Assay

The phospholipid concentrations were evaluated by the Barlett inorganic phosphate assay (16).

VIP Assay

VIP concentrations in liposomes were determined after treatment with sodium dodecyl sulfate 1% by a VIP ELISA assay kit (Peninsula Laboratories, Belmont, CA).

Morphological Evaluation

Liposomes were prepared for freeze-fracture according to standard techniques as reported previously (15). Briefly, drops of the liposome suspension were frozen in liquid-nitrogen cooled Freon 22, fractured using a Balzers BAF 301 freeze-etch unit at −115°C, and coated with platinum and carbon. The replicas were cleansed in a minimum of two changes of bleach (sodium hypochlorite), washed with distilled water, dried, collected on 200 mesh copper grids, examined and photographed with a JEOL 100CX transmission electron microscope at 80kv.

In Vivo Evaluation of VIP in SSL

Two types of *in vivo* experiments were performed to determine the vasorelaxant and hypotensive effects of VIP.

Effect on Arteriolar Diameter in the Hamster Cheek Pouch

The purpose of these experiments was to determine the bioactivity of VIP in SSL. Adult male golden Syrian hamsters (n = 9) were purchased from Sasco (Omaha, NE). Animals were prepared as previously described (3–5) and anesthetized with pentobarbital sodium via a cannulated femoral vein (2–4 mg/100 g body weight). A femoral artery was cannulated to record systemic arterial pressure and heart rate—that did not change significantly during the experiments—using a transducer and a strip-chart recorder (Model 260, Gould Instrument Systems Inc., Valley View, OH). The visualization of the microcirculation of the cheek pouch, an established animal model to investigate the vasoactive effects of neuropeptides *in situ*, was conducted as previously described (3–5). The inner-wall diameter of second order arterioles in the hamster cheek pouch was measured from the video display of the microscope image using a videomicrometer (VIA 100; Boeckeler Instruments, Tucson, AZ). In each animal, the same arteriolar segment was used to measure changes in diameter during the experiment. The hamster cheek pouch was first suffused with bicarbonate buffer for 30 min, and then with the test preparations (VIP in SSL Methods A and B; 1.4 ml) for 7 min in a non-systematic fashion.

Effect on Mean Arterial Pressure

The purpose of these experiments was to determine the duration and efficacy of VIP in SSL on mean arterial pressure after bolus i.v. injection. Adult male hamsters with spontaneous hypertension (n = 12) were obtained from the Canadian Hybrid Farms (Hall Harbour, Nova Scotia, Canada). The latter have been identified after cross-breeding of hamsters with hereditary cardiomyopathy and golden Syrian hamsters (4,17). Test preparations (VIP in SSL Method B, VIP only and liposomes only; 500 μl) were injected over 1 min in the femoral vein. Continuous anesthesia of the animals limited the monitoring of mean arterial pressure to 6 h.

Data Analysis

Data are expressed as mean ± SEM. Two-way analysis of variance and Student's *t* test with the Bonferroni correction for multiple comparisons were performed to compare responses between experimental groups. A p value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Development of a Suitable Method for a Bioactive SSL Formulation of VIP

Previous studies have evaluated the role of different factors on the circulation time of SSL, and found that ideally, the mean vesicle diameter should be under 200 nm, with PEG at a molecular weight of ~2,000 Da at a concentration of 5% (9–12). According to these reports, we chose a composition that mostly contained PC (50%), a common phospholipid of biological membranes, cholesterol (35%), which gives a required rigidity to the liposomal bilayers and has been recently shown to be preferable in SSL preparations (12), $PEG_{1,900}$-DSPE (5%) to obtain steric stabilization of the liposomes and small amounts of PG (10%), to provide some negative charge on the vesicle surface and probably promote the binding of the VIP molecules to membranes. The presence of PG in a SSL formulation was shown not to affect the half-life of the vesicles (10).

To prepare the formulation, we logically first tried to apply a method similar to the one previously used in our laboratory for the encapsulation of VIP in liposomes (2–4), with the addition of an extrusion step through 200 nm filters (Method A) to achieve the desired small size for the SSL (11). Figure 1 indicates that 0.1 nmol VIP in SSL prepared with the method A did not elicit an increase in arteriolar diameter significantly different from the one previously observed with 0.1 nmol VIP dissolved in saline, i.e. around 10% (3). However, encapsulation of VIP in conventional liposomes prepared with the same method, including sonication but without the 200 nm-extrusion, had been shown to enhance and prolong its effects *in situ* (3). These results suggested that three factors could be accounted for the loss of activity of VIP in SSL: the extrusion process, the new composition or the smaller size of the vesicles. Since SSL having the same composition and size prepared by method B retained the activity (see below), and that the VIP activity was similar in the presence and absence of trehalose, we believe that extrusion was responsible for the loss of bioactivity. As a matter of fact, it is not surprising that liposome size reduction techniques can be problematic in the case of encapsulated proteins and peptides due to their known sensitivity to harsh processing. The freeze/thaw-extrusion method has been frequently

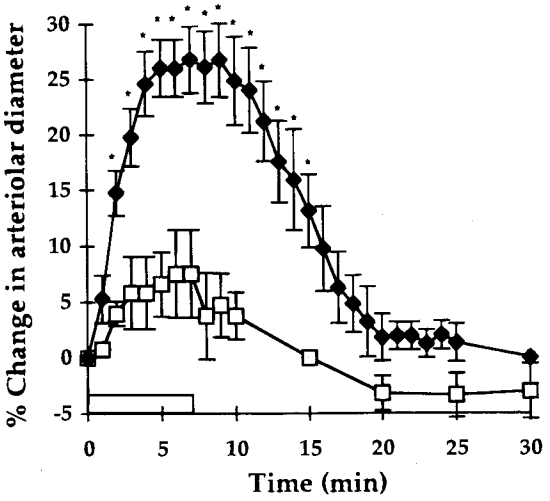

Fig. 1. Changes in arteriolar diameter during and following suffusion for 7 min (open bar) of VIP in SSL prepared by Method A (0.1 nmol, □) and by Method B (0.1 nmol, ◆). Values are mean ± SEM; Group A, n = 4 animals, Group B, n = 5 animals. *p < 0.05 compared to baseline.

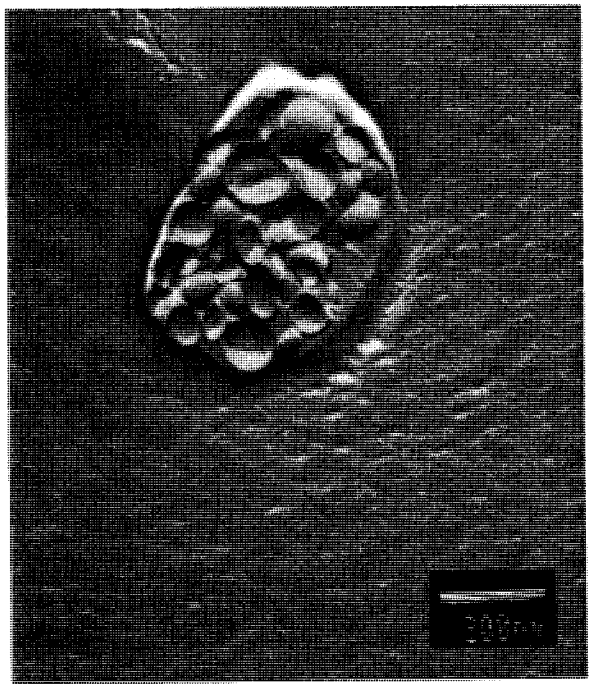

Fig. 2. Freeze-fracture micrograph showing the structure of VIP in SSL (Method B). × 40,000.

used in the past, in particular in the two other reported cases of loading of a protein or peptide in SSL. While the peptide vasopressin did not seem to be terribly affected by the extrusion process (13), interleukin-2 was reported to lose more than 25% activity after extrusion (14).

Since the peptide activity was almost totally lost in our first attempt, we decided to prepare VIP in SSL by avoiding the extrusion of the vesicles loaded with peptide. For this, we first extruded empty SSL of the same composition as above, and lyophilized this suspension in the presence of VIP and a cryoprotectant (Method B). During freeze-drying, VIP and phospholipid bilayers were in close contact and provided a significant amount (~30%) of passive drug loading. This new formulation of VIP in SSL (0.1 nmol) elicited a 28.3 ± 3% increase in arteriolar diameter from baseline, and lasted for another 9 to 16 minutes after suffusion was stopped (Figure 1; n = 4; $p < 0.05$). Hence, a biological effect similar to the one previously obtained with conventional liposomes was observed (3). This preparation (Method B) was chosen to be further tested.

Morphological Evaluation

In order to better understand the influence of the novel preparation method on the structural properties of the liposomes, freeze-etch electron microscopy was applied. The morphology of VIP in SSL (Method B) revealed multivesicular vesicles (Figure 2). Four random samples from the preparation were observed, and nearly all of the liposomes displayed a multivesicular structure. These results suggested that freeze-drying caused some fusion of the small pre-extruded SSL, forming vesicle-in-vesicle structures, which was confirmed by the observed increase in mean diameter from ~80 nm to ~250 nm. Similar fusion events were reported during the freeze-drying/reconstitution process of SSL in a recent study by Szucs and Tilcock (18). In our case, the formation of larger vesicles may have promoted the entrapment of VIP molecules inside the final liposomes, while retaining a rather small mean size and distribution required for long circulation times.

Therapeutic Effect of VIP in SSL in Hamsters with Spontaneous Hypertension

Once the formulation of VIP in SSL prepared by Method B was shown to be bioactive *in situ*, the next step was to test the efficacy of this new product for its systemic, long lasting therapeutic effect on hypertensive hamsters. The results are presented in Figure 3. After the injection of only 0.1 nmol VIP in SSL, there was a significant and gradual decrease in mean arterial pressure up to ~50%, that was obtained in the first 2.5

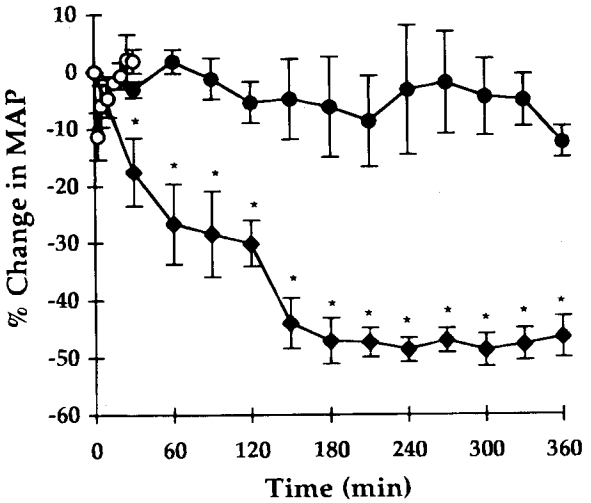

Fig. 3. Percentage change in mean arterial pressure after bolus i.v. injection of 0.1 nmol VIP in SSL (Method B, ◆), native VIP (○) and empty SSL (Method B, ●) in hamsters with spontaneous hypertension. Values are mean ± SEM; each group n = 4 animals. *p < 0.05 compared to baseline.

hours and which lasted throughout the observation period. Mean arterial pressure decreased from 150.2 ± 9.6 mmHg to 72.4 ± 8.3 mmHg, and stayed in this normal range for 6 h. No significant effects of empty SSL and aqueous VIP (0.1 nmol) were observed on mean arterial pressure. These data suggest that intravenously administered VIP in SSL successfully normalized the mean arterial pressure of hamsters with spontaneous hypertension for at least 6 hours. Moreover, the dose required to reach normal blood pressure was very low, only 0.1 nmol VIP. Our previous work had demonstrated a 30% decrease in mean arterial pressure of normotensive hamsters for a dose of 1 nmol VIP in conventional liposomes (2), i. e. 10 times more than what was administered in this study. However, this phenomenon may simply be attributed to a higher sensitivity of hamsters with spontaneous hypertension to VIP.

In summary, this study presents a novel and successful application of sterically stabilized liposomes as carriers for a peptide drug. Steric hindrance of the lipid surface did not eliminate the peptide drug activity, and provided a significant prolongation of its effects. We believe that this new product has a high potential to be used in the treatment of hypertension and other disorders involving impaired regional blood flow. However, the results of this study also raise many questions regarding the type of interactions between VIP and the liposomal membranes, the influence of PEG molecules on these interactions, the importance of the VIP conformation/activity relationship in the SSL delivery, the mechanisms mediating the physiological action(s) and signal transduction pathway(s) of this formulation, its dose dependence, pharmacokinetics and biodistribution patterns. Studies to answer some of these questions are presently ongoing in our laboratories.

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