Inhibition of Tracheal Vascular Extravasation by Liposome-Encapsulated Albuterol in Rats

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Purpose. To develop a liposome-based system for systemic delivery of anti-inflammatory drugs to airways and other inflamed tissues. Methods. Postcapillary venular gap junctions open during airway inflammation and allow fluid accumulation and permit molecules (e.g. complement, kininogen) to enter tissues, initiating inflammatory cascades. Beta-adrenergic agonists prevent inflammatory plasma extravasation, but because of their deleterious side effects, they are not used intravenously. When sterically stabilized “stealth” liposomes are injected iv, they remain in the circulation for long periods. Inflammatory mediators [e.g., substance P(SP)] open postcapillary venular gaps and allow liposomes and their contents to be deposited selectively in the inflamed tissue.

Results. We hypothesized that liposomes encapsulating a beta-adrenergic agonist, such as albuterol, would deposit selectively in inflamed airway tissue, where the drug would slowly leak out of the liposomes, resulting in closure of the gaps, thus preventing subsequent inflammatory extravasation. To test this hypothesis, we delivered albuterol-loaded liposomes iv in rats. Then we injected SP to open the venular gaps and allow accumulation of the drug-loaded liposomes in airway tissue. We examined whether this treatment resulted in inhibition of subsequent plasma extravasation induced by SP. The results indicate that liposome-encapsulated albuterol inhibits subsequent extravasation, presumably by leaking out of liposomes in airway tissue. This inhibition occurs for prolonged periods of time and with limited side effects compared to the effect of free albuterol.

Conclusions. We conclude that liposomes loaded with appropriate drugs, by migrating to inflamed tissue and subsequently inhibiting inflammatory cascades, may be of therapeutic value in inflammatory diseases.

KEY WORDS: beta-adrenergic agonist; endothelial gap junctions; antiinflammatory drug; liposome drug delivery.

INTRODUCTION

It has been established that sterically stabilized “stealth” liposomes circulate in blood for a long period of time with minimal localization in normal tissues except liver and spleen (1). However, when vascular permeability is increased by inflammatory disease or by delivering mediators that increase vascular permeability [e.g., substance P (SP)], stealth liposomes can cross the endothelial barrier and become localized in the inflamed tissue (2).

In experimental animals, increased vascular permeability leads to extravasation of plasma proteins, which can be inhibited by the administration of beta-adrenergic agonists (3–5). In the present study, we encapsulated the beta-adrenergic agonist albuterol into stealth liposomes and injected it intravenously. Because albuterol leaks out of liposomes relatively slowly, few side effects should occur due to free drug released into the circulation. The vascular permeability in the trachea was then increased by injecting SP iv, which allows the drug-loaded liposomes to “home” selectively into the tracheal tissue. This is possible because the tracheal endothelium contains SP receptors which, when stimulated, open endothelial gaps in tracheal vessels, allowing the extravasation of the albuterol-loaded liposomes.

Our basic premise was that albuterol would diffuse at a reasonable rate out of the liposomes in the tracheal tissue, resulting in relatively high concentrations of free albuterol in the inflamed tissue. We hypothesized that the released albuterol would close the endothelial gaps and prevent subsequent extravasation when a second iv injection of SP is made. The results of these studies showed that albuterol-loaded liposomes can inhibit subsequent tracheal vascular extravasation for prolonged periods of time and with minimal side effects compared to those of free albuterol.

MATERIALS AND METHODS

Materials

Hydrogenated soy phosphatidylcholine (HSPC) was purchased from Lipoid K.G., Lugwigshafen, Germany. Polyethylene glycol (molecular weight, 1900) derivative of distearoylphosphatidylethanolamine, sodium salt (mPEG-1900-DSPE) was synthesized as described (6) and purchased from Sygna, Inc., Liestal, Switzerland. Cholesterol was purchased from Croda, Inc., New York, NY; Albuterol (Salbutamol, Hemi-sulfate salt) from Sigma Chemical Company, St. Louis, MO; Substance P (SP), purity 98% (HPLC), from Bachem Bioscience Inc., Philadelphia, PA; Evans blue from Polysciences, Inc., Warrington, PA; Pentobarbital (nembutal sodium) from Abbott laboratories, North Chicago, IL. Other solvents and chemicals were of analytical grade.

Animals

Pathogen-free male rats of the F344 strain, 200-220 g body wt., were obtained from Simonsen Laboratories (Gilroy, CA). Experimental procedures followed in this study were approved by the Committee on Animal Research of the University of California San Francisco.

Preparation of Liposomes

Sterically stabilized liposomes composed of HSPC/cholesterol/mPEG-DSPE (weight ratio, 3:1:1) were prepared by thin
film hydration of mixed lipids with a solution of 250 mM ammonium sulfate (pH 5.5) at 60°C with rotation. The multilamellar vesicles (100 μmol total lipid/ml) thus formed were extruded through nucleopore polycarbonate filters (7), eight times each through 0.4, 0.1, and 0.05 μm pore size in turn. Extrusion was performed with a stainless steel extrusion cell from Lipex Biomembranes (Vancouver, Canada) under nitrogen pressure of 50 – 650 Psi. The extrusion system was maintained at 60°C. The liposome suspension was dialyzed against 10% sucrose, 5 mM sodium chloride for 3 days at 2 – 8°C, changing buffer once per day. The mean particle size of the liposomes was determined by dynamic light scattering using a sub-micron particle analyzer (Coulter model N4MD, Coulter Corporation, Miami, FL). The particle size (diameter) of liposomes was 100 – 130 nm.

Liposome-encapsulated albuterol contained the same lipid components and weight ratio as described above, and preparation of liposomes was also the same. Albuterol was dissolved in 10% sucrose solution at a concentration of 10 mg/ml. An equal volume of liposome and albuterol solutions were mixed, and final concentrations of total lipid and albuterol were ~50 μmol/ml and 5 mg/ml, respectively. The mixed liposome-albuterol solution was incubated at 60°C for 1 h, followed by rapid cooling on ice. To remove free albuterol, the liposome-albuterol solution was dialyzed against a buffer of 10% sucrose, 10 mM histidine, pH 6.5. Dialysis buffer was changed once per day for a period of 3 days. Albuterol-containing liposomes were filtered through a 0.45 μm Gelman acrodisc filter, then through a 0.2 μm filter into glass vials, sealed, and stored at 4°C. To examine drug loading efficiency, 400 μl of the sample after cooling on ice was loaded onto a Sephadex G-50 column of 15 cm x 0.1 cm, and then eluted with buffer containing 150 mM NaCl, 50 mM sodium acetate, 0.02% azide, pH 4.5. Thirty fractions of 1 ml were collected, diluted to 1:11 in 99% MEOH and 1% 1 N HCl. The UV absorbance of the diluted fractions was measured in a spectrophotometer at 281 nm. Albuterol-loaded liposomes appeared in fractions 4–8, and free albuterol appeared in fractions 11–20. The loading efficiency was ~60% of the original amount of drug added. The final preparation of albuterol-loaded liposomes contained ~36 μmol phospholipid/ml and ~1.9 mg albuterol/ml, where more than 99% of the drug is encapsulated. Phospholipid concentration was determined by phosphate assay as before (1) and albuterol by spectrophotometry at 28 nm.

**Assessment of Plasma Extravasation**

In this study, we examined the effects of albuterol, liposomes, and albuterol-loaded liposomes on substance P (SP)-induced plasma extravasation in rat trachea. Evans blue was used as a marker to assess plasma extravasation. All injections were i.v. (femoral vein).

**Liposome-encapsulated Albuterol vs. Liposomes Alone**

Seven groups of rats were used, 4 rats in each group. In group 1, the first injection was 0.9% saline. SP (3 nmol/kg body wt) was injected 3 min later. Evans blue (30 mg/kg) was injected 24 min after SP. Finally, the second dose of SP (3 nmol/kg) was injected 3 min after Evans blue. The animals were euthanized, and perfusion was performed with PBS buffer (pH 7.4) after another 8 min as follows: The chest was opened, a cannula was inserted into the ascending aorta through the left ventricle, the left atrium was incised, and perfusion was carried out for ~40 sec. Then the right atrium was incised, and perfusion was continued for another ~80 sec. Groups 2, 3, and 4 were the same as group 1, but saline was replaced with albuterol-containing liposomes. The doses of liposomal-albuterol for each group were 0.5, 1, and 2 mg/kg, respectively. Group 5 was also the same as group 1, except that liposomes alone (no albuterol) were used instead of saline. The amount of phospholipid given was ~36 μmol per kg of body wt (an amount equal to the phospholipid given in group 4). The difference between groups 1 and 6 was that the first injection of SP in group 1 was replaced with 0.9% saline in group 6. Group 7 was the same as group 6, but the albuterol-loaded liposomes (2 mg albuterol/kg) were used instead of the first saline.

**Time Course of Action of Albuterol-loaded Liposomes vs. Free Albuterol**

Injections of 0.9% saline, or albuterol (2 mg per kg of body wt) or albuterol-loaded liposomes (2 mg of liposomal albuterol/kg body wt) were followed by SP (3 nmol/kg) 3 min later. The second dose of SP (3 nmol/kg) was injected at various times: 0.5, 1, 2, and 4 h after saline, or albuterol or albuterol-loaded liposomes. Evans blue (30 mg/kg) was injected 3 min before the second dose of SP. The animals were perfused 8 min after the second injection of SP. Four rats each comprised of the groups of control and albuterol-loaded liposomes for each time point; in the free albuterol group only for 2 or 3 rats were studied for each time point, due to death of some rats after injection of albuterol prior to the completion of the experiments.

**Measurement of Evans Blue Extravasation in Rat Trachea**

After the perfusion, the trachea was cut below the larynx and above the bifurcation, and connective tissue was removed. Then, the anterior trachea was cut longitudinally, blotted with bibulous paper (Fisher Scientific Co. Pittsburgh, PA), and weighed. Evans blue in the trachea was extracted by immersion in 2 ml of formamide and shaking at room temperature overnight. The optical density of Evans blue in formamide was measured at a wavelength of 620 nm with a UV160U spectrophotometer (Shimadzu, Columbia, MD). The amount of Evans blue was determined by comparing the values to a standard curve of Evans blue and expressed in micrograms of Evans blue per g of trachea. This represents an assay for extravasated albumin, because Evans blue is known to bind to circulating albumin shortly after injection in blood.

**Measurement of Arterial Blood Pressure**

The effects of free albuterol and of albuterol-loaded liposomes on arterial blood pressure of rats were examined. The femoral vein was cannulated and connected to a pump. Intravenous injection of various drugs was carried out with the pump for 2 min: free albuterol, 2 mg/kg (N = 1 rat); albuterol-loaded liposomes, 0.5, 1, and 2 mg of albuterol/kg (N = 2 rats for each dose); liposomes alone: 36 μmol of phospholipid/kg (N = 1 rat). A femoral artery was cannulated and connected to a Statham transducer (model P23D, Statham, U.S.A.). Arterial blood pressure was recorded continuously for 30 min. In another experi-