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Enhanced oral absorption of insulin-loaded liposomes containing bile salts: A mechanistic study

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ABSTRACT

Liposomes containing bile salts (BS-liposomes) significantly enhanced the oral bioavailability of insulin (rhINS). However, the underlying absorption mechanisms have not been well understood yet. In this study, the transiting fate of the liposomes was first investigated using fluorescent imaging tools to confirm the effect of enhanced gastrointestinal stability. In order to obtain evidence of enhanced transcellular permeation, the interaction between BS-liposomes and the biomembrane was investigated in Caco-2 cell lines. BS-liposomes were found to be more stable in the gastrointestinal tract by showing prolonged residence time in comparison with conventional liposomes. BS-liposomes were significantly more effective for cellular uptake and transport of rhINS; and this effect was found to be size- and concentration-dependent. A good linear correlation was observed between the concentration of the liposomes and uptake/transport of rhINS. Confocal laser scanning microscopy visualization further validated the transcellular transit of BS-liposomes. The BS-liposomes showed little effect on cytotoxicity and did not induce apoptosis within 24 h investigation. It was concluded that BS-liposomes showed improved in vivo residence time and enhanced permeation across the biomemebranes. Mechanisms of trans-enterocytic internalization could be proposed as an interpretation for enhanced absorption of insulin-loaded liposomes.

1. Introduction

Administration of therapeutic peptide/protein drugs such as insulin via the oral route represents one of the greatest challenges in modern pharmaceutical technology. The presence of highly active enzymes in the gastrointestinal tract as well as the low permeability of the intestinal epithelia leads to very low bioavailability (Rekha and Sharma, 2013; Wang and Zhang, 2012). Therefore, various approaches have been investigated to enhance the oral delivery of these drugs. Among these, the use of nanocarrier systems such as liposomes (Abdallah et al., 2011; Dekel et al., 2010), mixed micelles (Wang et al., 2010), microemulsions (Sharma et al., 2010), microspheres (Zhang et al., 2011) and so on has shown great potential. The nanocarriers can protect peptides/proteins from enzymatic degradation to a certain extent and enhance intestinal absorption. The underlying mechanisms include M-cell uptake, transport through the paracellular pathway and transcellular permeation. The contribution of M cells to particle uptake is well acknowledged, but only to limited extent because the M cells comprise less than 10% of the intestinal epithelial cell population (Anne-Marie et al., 2000; Sheetal et al., 2007; Thejani et al., 2010). However, the transcellular uptake through enterocytes remains poorly understood. Improved understanding, either quantitatively or qualitatively, of the interaction between the vehicles and the biological environment helps to design more efficient carriers for oral delivery of peptides/proteins.

In our previous studies, a bile salt-reinforced liposome (BS-liposomes) system composed of soybean phosphatidylcholine and three types of bile salts (sodium glycocholate, sodium taurocholate and sodium deoxycholate) was developed for the oral delivery of insulin (Niu et al., 2011, 2012). The prepared BS-liposomes showed good protection of insulin against enzymatic degradation by pepsin, trypsin and α-chymotrypsin in vitro and a sustained hypoglycemic effect for 24 h with oral bioavailability of 10.2% in diabetic rats. BS-liposomes present enormous potential as an effective carrier system for oral delivery of insulin. Some studies in the field of oral vaccination (Shukla et al., 2008; Singh et al., 2004) revealed that incorporation of bile salt in liposomal formulation could stabilize the membrane against the detrimental effects of bile acids in the gastrointestinal tract (GIT) (Schubert et al., 1983). Besides,

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the presence of bile salts possibly enhances the internalization of BS-liposomes due to their absorption-enhancing effect (Niu et al., 2012).

In this study, we first investigated the transiting fate of recombinant human insulin (rhINS)-loaded BS-liposomes after oral administration using imaging tools. In order to understand the absorption mechanisms, the interaction between BS-liposomes and biological membranes was evaluated in the well-established human intestinal Caco-2 cell lines. Particle size and concentration of BS-liposomes, which affected the uptake and permeability of rhINS, was also examined. In addition, since bile salts were reported to be toxic when they are used as penetration enhancers (Saettone et al., 1996), preliminary evaluation of cytotoxicity was carried out to illuminate the safety of BS-liposomes as an effective oral delivery system.

2. Materials and methods

2.1. Materials

rhINS (27 IU/mg) was provided by Novo Nordisk (Copenhagen, Denmark). Sodium glycocholate (SGC) and RNase were purchased from Amresco (Solon, OH, USA). Soybean phosphatidylcholine (SPC) and cholesterol (CH) were supplied by Lipoid (Germany). Sodium taurocholate (STC), sodium deoxycholate (SDC) and NHS-fluorescein were obtained from Sigma (St. Louis, MO, USA). Sephadex G-50 was purchased from Pharmacia (USA). ELISA kit for human rhINS was purchased from Westang Bio-Technology Co. Ltd. (Shanghai, China). Rabbit anti ZO-1, fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG and tetramethylrhodamine B isothiocyanate labeled Phalloidin were obtained from Sigma (St. Louis, MO, USA). Goat serum was purchased from Yixin Bio-Technology Co. Ltd. (Shanghai, China). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) was purchased from Sigma (St. Louis, MO, USA). Water was deionized using a Milli-Q system from Millipore (Billerica, MA, USA). All other reagents and chemicals were of analytical grade and used without further purification.

Caco-2 cells were obtained from ATCC (MD, USA). Mycoplasma free cells of passage 30–50 were used throughout. The cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% (v/v) fetal calf serum (Gibco, USA), 1% (v/v) nonessential amino acids, 1% (w/v) L-glutamine, and 100 mg/ml streptomycin (Gibco, USA). The cells were maintained at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity.

Male Wistar rats (200 ± 20 g) were used throughout the study. We have gotten approval from the ethics review board of Novo Nordisk and have followed the principles outlined in the Declaration of Helsinki for all animal experimental investigations.

2.2. Preparation and characterization of rhINS-loaded BS-liposomes

BS-liposomes (containing SGC, STC or SDC) were prepared by a reversed-phase evaporation method according to our previous report (Niu et al., 2011). Briefly, soybean phosphatidyl choline (SPC) and bile salts (SGC/STC/SDC) were dissolved in 5 mL absolute ether with a molar ratio of 4:1, into which 1 mL rhINS solution in citric–Na_2HPO_4 buffer (4 mg/mL, pH = 3.0) was added. The mixture was ultrasonicated in a water bath to form w/o emulsion, which was subsequently evaporated under reduced pressure with a rotating speed of 50 rpm at 30 °C for 20 min to remove the organic solvent. After that, 10 mL citric acid–Na_2HPO_4 buffer (pH 5.6) was added to hydrate the lipid until a homogeneous dispersion with pH around 5.6 formed. Finally, this dispersion was extruded through a high-pressure homogenizer (AH 100 D, ATS Engineering Inc.) to obtain liposomes containing different kinds of bile salts with different sizes. Conventional liposomes were prepared similarly using CH instead of bile salts.

The particle size was evaluated by dynamic light scattering instrument (NICOMP 380 ZLS Particle Sizing Systems, USA). rhINS entrapment efficiency was measured by using molecular exclusion chromatography with a 30 cm-long Sephadex G50 column as described in the previous study (Niu et al., 2011).rhINS was measured by an HPLC-UV method as described before (Niu et al., 2011) with a C18 column (Zorbax, 5 μm, 4.6 mm × 150 mm, Agilent) maintained at 25 °C, an UV detector set to 220 nm and a mobile phase composed of 26% acetonitrile and 74% water (adjusted to pH 2.5 by H_3PO_4).

2.3. Fluorescence labeling of rhINS

For cellular uptake study, fluorescein labeled rhINS was synthesized as described previously (Clausen and Bernkop-Schnürch, 2000) with minor changes. Briefly, 1 mg rhINS was dissolved in 2 mL pH 8.0 PBS, into which NHS-fluorescein in dimethyl sulphoxide (DMSO) was gradually added in volumes of 30 μL, with rhINS to fluorescein mole ratio of 1:2. The mixture was incubated for 2 h at 25 °C with gentle agitation. The resulting F-rhINS was washed three times with PBS by ultrafiltration using a membrane (3000 D cutoff) at a centrifugal speed of 1100 × *g*. For in vivo imaging study, the probe carbocyanine IR 783 was used because of its high sensitivity, low tissue irradiation and relatively low cost. It fluoresces in the near-infrared (NIR) wavelength range (700–900 nm) where autofluorescence and light absorption of tissue and fluid are low. Moreover, the NIR light can penetrate several centimeters into rat tissues (Chen et al., 2010; Frangioni, 2003). IR 783 was synthesized according to a reported method (Li et al., 2006). rhINS-IR 783 conjugation was carried out as described above but substituting fluorescein with IR 783 at rhINS to IR 783 mole ratio of 1:5.

2.4. Intra-gastrointestinal fate of BS-liposomes by in vivo luminescence imaging

Oral absorption of rhINS-liposomes depends highly on their ability to withstand gastrointestinal degradation by enzymes and gastric acid. To protect rhINS and to prolong its residence in the GIT might improve its absorption. Herein, in vivo imaging method was applied to identify the gastrointestinal residence profiles after oral administration of rhINS-loaded liposomes. BS-liposomes and controls with rhINS concentration of 20 IU/kg (2 IU/mL, 2 mL/rat) were given by gavage to Wistar rats followed by anesthesia immediately by intraperitoneal injection of 30 mg/kg pentobarbital sodium solution. The animals were subsequently cut open in the abdomen to expose the GIT and placed in a supine position in a light-tight chamber. In vivo luminescence imaging was performed with an IVIS 200 imaging system (Xenogen, Alameda, CA, USA) linked to Living Image 3.1 software (Xenogen) (Hühn et al., 2010). This system provides high signal-to-noise ratio images of fluorescence signals emerging from within live animals (Chen et al., 2010). A reference image was obtained firstly under natural light illumination. Then images were monitored 15, 30, 60, 120 and 240 min after oral administration with optical excitation at 700 nm and emission at 950 nm. At the same time point after imaging, 0.2 mL blood was obtained from the tail vein and rhINS concentration in blood serum was measured using ELISA.