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Calcium phosphate-PEG-insulin-casein (CAPIC) particles as oral delivery systems for insulin

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Abstract

An oral delivery system for insulin was developed and functional activity was tested in a non-obese diabetic (NOD) mice model. Calcium phosphate particles containing insulin was synthesized in the presence of PEG-3350 and modified by aggregating the particles with caseins to obtain the calcium phosphate-PEG-insulin-casein (CAPIC) oral insulin delivery system. Single doses of CAPIC formulation were tested in NOD mice under fasting or fed conditions to evaluate the glycemic activity. The blood glucose levels were monitored every 1–2 h for 12 h following the treatments using an ACCU CHECK blood glucose monitoring system. Orally administered and subcutaneously injected free insulin solution served as controls in the study. Based on the results obtained we propose that: (1) the biological activity of insulin is preserved in CAPIC formulation; (2) insulin in CAPIC formulations, but not the free insulin, displays a prolonged hypoglycemic effect after oral administration to diabetic mice; (3) CAPIC formulation protects insulin from degradation while passing through the acidic environment of the GI track until it is released in the less acidic environment of the intestines where it can be absorbed in its biologically active form; (4) CAPIC formulation represents a new and unique oral delivery system for insulin and other macromolecules.

Keywords: Oral drug delivery; Insulin; Diabetes; Oral insulin formulation; Sustained release drug delivery; Calcium phosphate particles

1. Introduction

Insulin is an essential hormone produced by the pancreas. Diabetes develops when the pancreas does not produce and secrete enough of this hormone, which is required for the normal sugar, protein, and fat metabolism of the body. Type I diabetes is characterized by its onset in young age, while Type II diabetes develops rather slowly and onsets in adult age. At present there is no cure for diabetes. People with Type I diabetes mellitus (insulin-dependent) depend on exogenously administered insulin for survival. The most common form of insulin therapy is twice-daily subcutaneous injection (via syringe, pen with needle and cartridge) of the hormone at 50–100 IU/injection. Commercial availability of various injectable insulin products, such as rapid-, short-, intermediate-, and long-acting forms, helps customizing insulin therapy for individual patient need. However, frequent-injection remains a painful treatment leading to needle phobia and patient noncompliance. Global interest to develop non-invasive, alternative methods to deliver macromolecule drugs, such as insulin, continues to grow. Oral and, in recent years, pulmonary routes (Trehan and Ali, 1998; Carino and Mathiowitz, 1999; Skyler et al., 2001) have emerged

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as potential alternatives to parenteral administration. Although oral administration of drugs is the most convenient way to treat patients, it has been less than ideal for peptide and protein drugs, such as insulin, due to their sensitivity to hydrolysis and enzymatic degradation, rapid clearance from the site of deposition, and poor absorption through epithelial membrane in the small intestines (Loehry et al., 1970). For the development of oral insulin, some investigators used absorption enhancers to increase intestinal permeability; others tried inclusion within liposomes (Coudhari et al., 1994) or polymeric particles (Michel et al., 1991; Hosney et al., 1998; Damage et al., 1988) to protect the insulin from proteolytic degradation. In recent years, a number of potential oral insulin formulations have been developed. Generex's buccal insulin mouth spray, Emisphere's SNAC-insulin capsule formulation, and Nobex's hexyl-PEG-modified peroral insulin are currently in various stages of clinical development (Abbas et al., 2001; Modi, 2001; Price, 2001).

Previously we reported pre-clinical studies indicating that CAP particles (Bell et al., 2002) are non-toxic, and cause no adverse reaction at the site of administration when given by injection, orally or intramuscularly as carriers for vaccine antigens (He et al., 2000). In a Phase I double-blind, placebo-controlled human clinical study, a single subcutaneous dose of CAP was injected into healthy volunteers. Results showed that CAP was safe to use in humans, it was well tolerated, and no difference in side-effect profile between CAP and placebo was observed. We also demonstrated the potential for calcium phosphate-PEG-Insulin (CAP-PEG-Ins), or insulin containing CAP particles (CAPI particles; 1–5 μm) as a pulmonary insulin delivery system (Morcol et al., 2001a) When CAPI (12.5 U/kg) was delivered into the lungs via intratracheal (IT) administration, fasted glucose levels were reduced by 75–80% within the first 1 h of treatment and the glycemic effect of insulin was increased from 4 to 5 h to at least 12 h relative to insulin solution. We further hypothesized that, when CAP-PEG-Ins particles are coated with casein, insulin will be protected in the acidic environment of the stomach when administered orally, and would be transported to the intestines where it would be absorbed into blood the stream (Morcol and Bell, 2001b). Fig. 1 illustrates our CAPIC oral insulin delivery system. In this study, the functional activity of our novel oral insulin formulation, CAPIC, was evaluated in a diabetic mice model.

2. Materials

Recombinant human insulin, PEG-3350, and bovine casein were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Calcium chloride ($CaCl_2$), sodium citrate ($Na_3C_6H_5O_7$), and sodium dibasic phosphate (Na_2HPO_4) were obtained from Fischer Scientific (Pittsburgh, PA). The Accu-Check glucose monitoring kit and glucose strips were purchased from a local drug store. Protein assay kit was obtained from Bio-Rad (Hercules, CA).

2.1. CAP-PEG-Ins (CAPI) particle preparation

One volume of insulin solution from a stock solution of 20 mg/ml in 0.01N HCl was mixed with 1% PEG-3350 to a final volume of 1 mg/ml. One volume

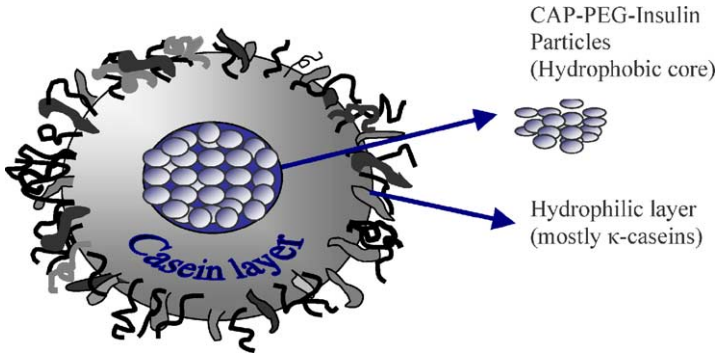

Fig. 1. Schematic of CAPIC^{BioSante} oral insulin delivery system.

$CaCl_2$ (125 mM), 0.2 volume $Na_3C_6H_5O_7$ (156 mM), and 1 volume Na_2HPO_4 (125 mM) were added, simultaneously to initiate the calcium phosphate (CAP) formation. The resulting particle suspension was stirred for 48 h at room temperature (rt) and sonicated to obtain stable particles in the size range of 2–4 μm. Free (residual) reaction components were separated from the particle suspension by centrifugation. Particles containing bound-insulin were washed and re-suspended in distilled water. Washed particles, which are stable for at least 6 weeks at 4 °C, were stored refrigerated until ready to process further for casein coating. Control CAP particles were prepared similarly with the exclusion of PEG and insulin from the original reaction mixture. The mean particle sizes of CAPI and control CAP particles were determined by photon correlation spectroscopy using Beckman Coulter N4 Plus submicron particle sizer.

2.2. Measurement of insulin loading capacity

Small aliquots of CAP-PEG-Ins particle suspension were centrifuged at 4000 × *g* for 15 min. Particle pellet containing bound-insulin was dissolved in 0.01N HCl to elute bound-insulin. The Bradford's method (Price, 2001) was used to determine the insulin concentration in solubilized particle fraction. Insulin loading capacity and the loading efficiency was calculated using the following equations:

$$\text{Insulin loading percentage (w/w)} = \frac{M_{\text{bound}}}{W_{\text{particle}}} \times 100 \quad (1)$$

$$\text{Loading efficiency (\%)} = \frac{M_{\text{bound}}}{W_{\text{theoretical}}} \times 100 \quad (2)$$

where M_{bound} is the amount of insulin (mg) eluted from the particles (bound-insulin), M_{particle} is the amount of particle (mg) utilized for insulin binding, and $M_{\text{theoretical}}$ is the theoretical loading amount of insulin originally added in the reaction mixture.

2.3. Preparation of oral CAPIC (CAP-PEG-Ins-Cas)

Two milligrams/milliliter suspension of bovine casein (Sigma) was prepared in 10 mM phosphate buffer, pH 8. Washed CAPI particle pellets containing insulin were dispersed in casein suspension to a final particle concentration of 2.5 mg solid/ml. The suspension was rotated for 2 h at room temperature and stored overnight at 4 °C for maximum casein precipitation. Casein-coated particles were collected and washed by centrifugation and lyophilized to dryness at 27×10^{-3} mbar and −48 °C. Dry weight was determined and unit dose of insulin was estimated from the following equation:

$$\text{U Ins/mg CAPIC} = \left(\frac{M_{\text{bound}}}{W_{\text{CAPI particle}}}\right)\left(\frac{W_{\text{CAPI particle}}}{W_{\text{CAPIC particle}}}\right) \times (28\,\text{U/mg}) \quad (3)$$

where $M_{\text{bound}}/W_{\text{CAPI particle}}$ is the weight ratio of insulin originally incorporated in CAPI and $W_{\text{CAPI particle}}/W_{\text{CAPIC particle}}$ is the weight ratio of CAPI initially used to produce CAPIC. Insulin (Sigma) used in the formulations contained approximately 28 U insulin/mg.

2.4. Scanning electron microscopy

The morphology of CAP and CAPIC particles were analyzed using scanning electron microscopy (SEM). Small volumes (20–30 μl) of particle suspensions were air-dried on double-sided adhesive tape on metal stubs of the instrument and scanned at 3–10 kV.

2.5. In vitro drug release from CAPIC

To characterize the pH-dependent insulin release profiles of CAPIC, known amounts of particles were incubated at 37 °C for 0.5, 1, 2, and 3 h either in glycine buffer at pH 3 or in phosphate buffer at pH 6.5. The dispersions at each time were centrifuged through a 50 kDa size exclusion filters to fractionate free insulin and casein from intact particles. Insulin and casein in the filtrate fraction was separated using 12 kDa filters. Filtrate from 12 kDa filters was analyzed for insulin.

2.6. In vivo experiments

Female non-obese diabetic (NOD) mice (13–15 weeks of age) were divided into groups of five animals. Animals were either fasted (four groups) for

Table 1
Treatment schedule

Group	Delivery	Formulation	Insulin dose (U/kg)
1 Fed	Untreated	–	–
2 Fed	Oral	CAPIC	100
3 Fed	Oral	Insulin solution	100
4 Fasted	Untreated	–	–
5 Fasted	Oral	CAPIC	100
6 Fasted	Oral	Insulin solution	100
7 Fasted	s.c.	Insulin solution	12.5

15 h before the treatments and remained fasted during the experiment or remained on normal feeding regimen (three groups) throughout the blood-sampling period (Table 1). Pre- and post-fasting blood glucose levels were determined using an Accu-Check AdvantageTM glucose monitoring kit.

A single dose of 100 U/kg CAPIC suspension or insulin solution was administered directly into the stomachs of fed or fasted animals using a ball-tipped gavage needle. Blood samples were taken from the orbital sinus every 0.5–2 h for 12 h to determine the blood glucose levels. Subcutaneously (s.c.) injected insulin solution (12.5 U/kg) was used for comparison. The protocol used in the study was approved by the local IACUC.

3. Results and discussion

3.1. Physical properties

Scanning electron microscopy analysis of plain CAP particles is shown in Fig. 2, which reveals a spherical morphology with an average particle size of 600 nm, which has also been confirmed by measurements using the N4 Plus submicron particle sizer. Particles appeared to be slightly elongated after formulation with insulin and further coated with casein to generate CAPIC oral insulin delivery system (Fig. 3). It is important to note that, final particle size, either before or after the casein coating, could be manipulated to the desired size range by adjusting the sonication time.

Insulin loading capacity of CAP particles in the presence of PEG was about 60% (w/w). Without the PEG, only about 10% insulin could be incorporated in the particles. Our previous investigations using hydrophobic interaction chromatography and Zeta potential measurements indicated that particles synthesized in the presence of PEG presents less negative surface charge than the ones without the PEG. Considering that insulin is a negatively charged protein, we speculate that PEG provides a less negatively charged surface and more hydrophilicity to CAP facilitating more protein adsorption.

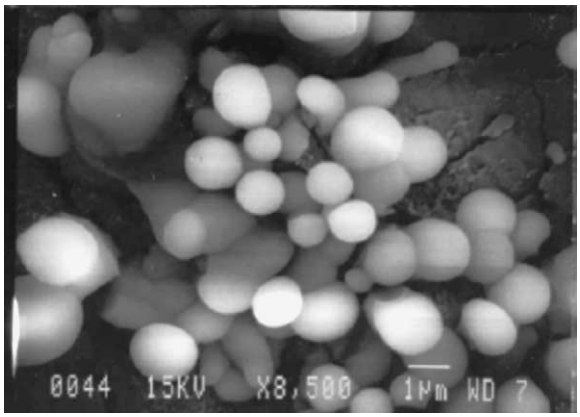

Fig. 2. SEM analysis of plain CAP particles.

3.2. In vitro insulin release

Percentage of insulin release from CAPIC particles at 37 °C as a function of pH and duration of exposure is shown in Fig. 4. As we predicted, there was minimum insulin leakage from the particles in a pH 3 environment, most likely due to further aggregation of casein at acidic pH. At the end of 3 h incubation at 37 °C, only about 12% insulin was released into the medium from CAPIC. On the other hand, insulin release was significantly higher at pH 6.5, such as in the intestines, and the profile suggested a sustained release mechanism (25–46% within 0.5–3 h). We anticipate that more insulin would be released from the particles as factors other than pH would also contribute particle degradation in the natural intestinal flora.

3.3. Effects of oral CAPIC on fasted-glycemia

Oral administration of 100 U/kg CAPIC reduced the fasted blood glucose levels by 80% within the first

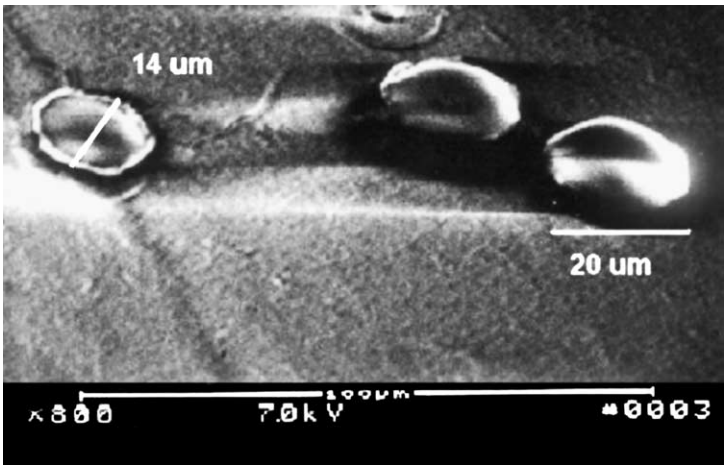

Fig. 3. SEM analysis of calcium phosphate-peg-insulin-casein (CAPIC) oral delivery system.

1 h of the treatment and remained reduced for 12 h. With oral insulin solution (without the CAP or PEG), glucose levels were reduced only slightly (20%) for a shorter time period and gradually returned to the levels of control within 5 h. Glycemic effect of 100 U/kg CAPIC on fasted blood glucose levels by the oral route was comparable to that of 12.5 U/kg insulin solution by s.c. injection (Fig. 5). Due to the limitations of collecting enough blood from mice, in vivo insulin absorption from the particles and PK/PD analysis could not be evaluated with this animal model. We have recently conducted oral CAPIC studies in fasted normal rats and results will be reported separately when completed.

3.4. Effects of oral CAPIC on fed-glycemia

Oral administration of 100 U/kg CAPIC to fed diabetic mice resulted in about 50% reduction of the initial glucose levels within the first 3 h of the treatment (Fig. 6). Glucose returned to the control levels within 5 h. An identical dose of insulin solution (with no CAP) had no significant effect on blood glucose levels.

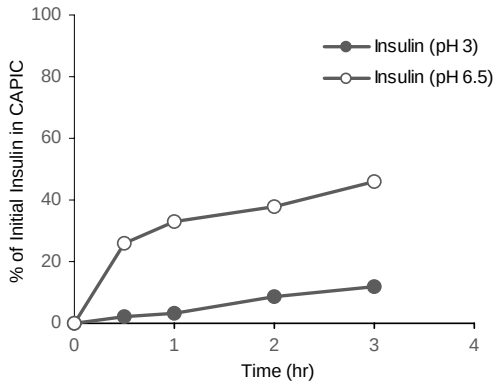

Fig. 4. In vitro insulin release from CAPIC in pH 3 and 6.5 at 37 °C.

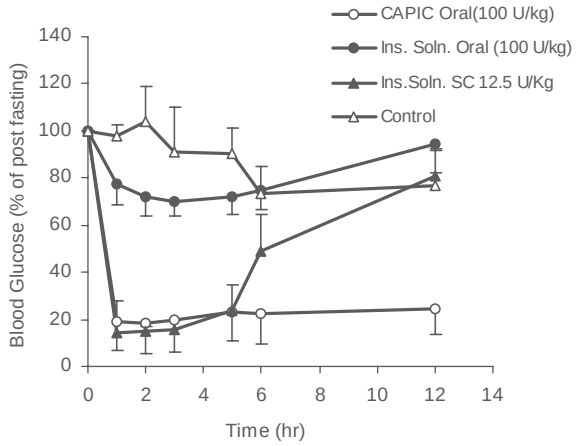

Fig. 5. Glycemic effect of a single oral dose of CAPIC in fasted-diabetic mice.

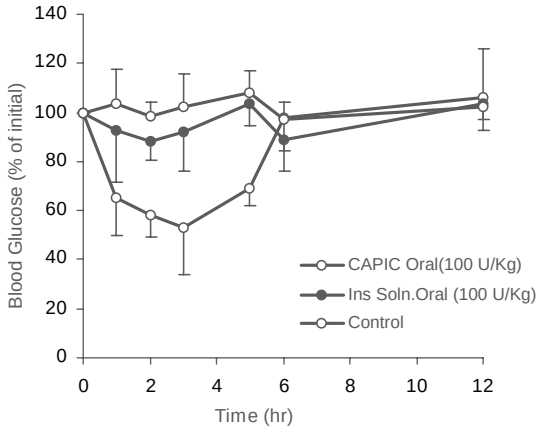

Fig. 6. Glycemic effect of a single oral dose of CAPIC in fed-diabetic mice.

4. Conclusion

We previously demonstrated that BioSante's proprietary calcium phosphate particles could be used to prepare casein-free whey fractions from transgenic milk based on the principle of chelating natural milk calcium to isolate recombinant proteins (Morcol and Bell, 2001b; Morcol et al., 2001c). The basic principle of the study was deconstructing casein micelles from milk, by chelating the calcium in the micelles' calcium phosphate core, and subsequently reconstructing them around our CAP particles freeing any recombinant protein trapped inside the original micellar structures. We used a similar approach in this study and used CAPI to form micelles from casein solution thus coating CAPI with caseins (CAPIC). We hypothesized that CAPIC would have a more collapsed structure in acid environments such as in the stomach, since acid precipitation is traditionally used in milk industry to aggregate caseins. Casein around the particles would start relaxing as pH increases; such as in the intestines, thus particles would start degrading and releasing insulin to the surrounding tissues and eventually into the blood stream. We generated CAPIC particles based on this hypothesis and tested the potential to deliver insulin via the oral route in mice. Our results obtained from the in vitro drug release experiments indicated that our hypothesis of protecting insulin from the harsh acidic environment of the stomach by coating the insulin containing particles with casein was valid. Furthermore, data from in vivo experiments indicated that insulin's functional activity is preserved in CAPIC formulation and CAPIC has the potential to deliver biologically active insulin orally. Thus, we believe other therapeutic proteins can be formulated similarly for oral route.

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