European Journal of Pharmaceutics and Biopharmaceutics xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research Paper

Effect of different intestinal conditions on the intermolecular interaction between insulin and cell-penetrating peptide penetratin and on its contribution to stimulation of permeation through intestinal epithelium

Noriyasu Kamei [a], Yukina Aoyama [a], El-Sayed Khafagy [a,b], Mao Henmi [a], Mariko Takeda-Morishita [a,*]

[a] Laboratory of Drug Delivery Systems, Faculty of Pharmaceutical Sciences, Kobe Gakuin University, 1-1-3 Minatojima, Chuo-ku, Kobe, Hyogo 650-8586, Japan
[b] Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmacy, Suez Canal University, Ismailia 415-22, Egypt

ARTICLE INFO

Article history:
Received 26 February 2015
Revised 28 April 2015
Accepted in revised form 30 April 2015
Available online xxxx

Keywords:
Oral absorption
Insulin
Cell-penetrating peptide
Penetratin
Intermolecular interaction
Caco-2 cells

ABSTRACT

Our recent studies have shown that the coadministration of cell-penetrating peptides (CPPs) is a potential strategy for oral delivery of peptide- and protein-based biopharmaceuticals. The intermolecular interaction between drug and CPP is an essential factor in the effective delivery of these drugs, but the characteristics of the interaction under the conditions of the intestinal lumen remain unknown. In this study, therefore, we examined the characteristics of binding of the amphipathic CPP penetratin to insulin and the efficiency of its enhancement of epithelial insulin transport at different pH and in simulated intestinal fluids (SIFs). The binding between insulin and penetratin was pH dependent and particularly decreased at pH 5.0. In addition, we clarified that the sodium taurocholate (NaTC) present in two types of SIF (fasted-state SIF [FaSSIF] and fed-state SIF [FeSSIF]) affected binding efficiency. However, the permeation of insulin through a Caco-2 cell monolayer was significantly facilitated by coincubation with L- or D-penetratin at various pH values. Moreover, the permeation-stimulating effect of L-penetratin was observed in FaSSIF containing NaTC and lecithin, but not in 3 mM NaTC solution, suggesting that the presence of lecithin was the key factor in maintaining the ability of penetratin to enhance the intestinal absorption of biopharmaceuticals. This report describes the essential considerations for *in vivo* use and clinical application of a CPP-based oral delivery strategy.

1. Introduction

Peptide and protein-based biopharmaceuticals have provided an advantage in the pharmacotherapy of several diseases and disorders [1]. However, most of them are injectable formulations, which may sometimes complicate their use by patients. The development of noninvasive forms of biopharmaceuticals to improve patients' quality of life and encourage compliance remains a challenge [2–4]. One of the major factors hampering the development of noninvasive forms of biopharmaceuticals is their poor permeability through biological membranes [5–8]. For instance, although oral administration is the most attractive route for patients, intestinal membranes consisting of epithelial cell layers function as permeability barriers and limit the influx of drugs into systemic circulation. Despite great efforts to overcome the difficulties of oral administration, no perfect strategy has yet been established. Insulin is a typical peptide drug that is used to treat diabetes, and to date a range of formulations have been developed, including super rapid, rapid, intermediate, extended action and mixed forms. Although a noninvasive inhaler form of insulin was transiently on the market, this was rejected because of concerns related to usability, cost, and safety. Even though new inhaler form of insulin was currently approved by FDA, the oral form of insulin is still best option for patients in point of endogenous secretion-mimetic behavior in addition to convenient usability.

We have recently proposed an effective and safe approach for oral delivery of peptide and protein drugs using cell-penetrating peptides (CPPs) [9,10]. CPPs, which can be effectively internalized into various cells, have been often used as useful tools for intracellular delivery of peptides, proteins, nucleic acids and nanoscaled carriers such as liposomes [11–13]. We previously demonstrated that the absorption of insulin from the intestine was significantly enhanced by coadministration of the CPPs octaarginine (R8) or penetratin or its analogues [9,14]. The efficiency of absorption enhancement by CPPs differed between CPPs consisting of L- and D-form of amino acids. The highest dose (2 mM) of L-penetratin increased the relative bioavailability of insulin to 35% [9]. Most

* Corresponding author. Tel.: +81 78 974 4816; fax: +81 78 974 4820.
E-mail address: mmtakeda@pharm.kobegakuin.ac.jp (M. Takeda-Morishita).

http://dx.doi.org/10.1016/j.ejpb.2015.04.030
0939-6411/

importantly, our previous study using surface plasmon resonance (SPR) analysis demonstrated that the intermolecular interaction between insulin and CPPs was essential for the enhanced absorption [14–16]. Insulin may form complexes with R8 and penetratin via electrostatic force and electrostatic/hydrophobic forces, respectively. That is, the CPPs can deliver into cells the cargo drugs that are attached to CPPs via electrostatic or hydrophobic interactions.

We focused on the possibility that the insulin–CPP interaction may vary under the conditions in the intestine, because luminal parameters including pH and fluid constituents differ along the intestine. Changes in the intermolecular interaction between drug and CPP may diminish the enhancement of absorption mediated by CPPs. In the present study, we assessed using SPR the characteristics of the binding between insulin and CPPs, in particular penetratin that is *Drosophila* antennapedia-derived amphipathic peptide [17,18], in buffers having different pH values and in simulated intestinal fluids (SIFs). Furthermore, to investigate the contribution of the altered binding characteristics to the stimulatory effect of CPPs on epithelial insulin uptake, transcellular insulin transport assays using Caco-2 cell monolayers were performed under different pH and SIF conditions. By analyzing the relationship between insulin–CPP interaction and the enhancement of absorption mediated by CPPs under the simulated conditions of the intestinal lumen, more precise understanding about the ability and limitation of CPPs to facilitate the intestinal absorption of insulin would be obtained. The information is essential for developing an optimal dosage form of oral insulin by using CPPs.

2. Materials and methods

2.1. Materials

Recombinant human insulin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-penetratin and D-penetratin (RQIKIWFQNRRMKWKK and rqikiwfqnrrmkwkk, respectively) were synthesized by Sigma–Genosys, Life Science Division of Sigma–Aldrich Japan Co. (Hokkaido, Japan). Premixed lecithin/sodium taurocholate (NaTC) reagent was purchased from Celeste Corp. (Tokyo, Japan). NaTC was purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). Carboxymethyl dextran (CM5)-coated sensor chips were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Human colon adenocarcinoma-derived Caco-2 cell line was purchased from the American Type Culture Collection (Rockville, MD, USA) at passage 18. Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, nonessential amino acids (NEAA), antibiotic mixture (10,000 U/mL penicillin, 10,000 μg/mL streptomycin, and 29.2 mg/mL L-glutamine in 10 mM citric acid-buffered saline), 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA), and Hanks' balanced salt solution (HBSS) were purchased from Gibco Laboratories (Lenexa, KS, USA). Fetal bovine serum (FBS) was purchased from Biowest (Nuaillé, France). 2-Morpholinoethanesulfonic acid (MES) monohydrate and 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) were purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of analytical grade and are commercially available.

2.2. Preparation of buffered solutions and SIFs

To prevent the adsorption of insulin and penetratin to the tube surfaces, methylcellulose (MC) was added at 0.001 w/v% to all the HBSS used in this study. For comparison of different pH, HBSS with 10 mM MES or 10 mM HEPES was adjusted to a final pH of 5.0, 6.0, 6.5, 7.0, or 8.0. The SIFs were prepared based on the method reported originally by Galia et al. [19], and in this study, the compositions of fasted-state SIF (FaSSIF) and fed-state SIF (FeSSIF) were partially modified as listed in Table 1 with the aim of reducing the loss of integrity of the Caco-2 cell monolayer.

2.3. Preparation of the insulin and penetratin stock solutions

To prepare the insulin stock solution (10×), specific amounts of recombinant human insulin were dissolved in 100 μL of 0.1 M HCl. The insulin solution was diluted with 0.8 mL of HBSS/0.001% MC at the appropriate pH, and was then normalized with 100 mL of 0.1 M NaOH. To prepare the penetratin stock solution (10×), specific amounts of L- or D-penetratin were dissolved in HBSS/0.001% MC at the appropriate pH. In the binding assay and transcellular transport assay, these stock solutions were diluted 10 times to achieve the final concentrations.

2.4. Surface plasmon resonance-based binding assay

The intermolecular interaction between insulin and L- or D-penetratin was analyzed by SPR (Biacore X-100, GE Healthcare UK). To measure the binding of L- or D-penetratin to insulin, insulin was immobilized at the carboxymethyl dextran surface of a CM5 sensor chip using amine coupling. For the immobilization procedure, insulin was diluted to a final concentration of 50 μg/mL using acetate buffer at pH 4.5, and immobilized on the chip surface in separate flow cells at 10 μL/min for 7 min. The reference surfaces were prepared by amine coupling activation followed by immediate deactivation. For binding measurements, different concentrations of L- or D-penetratin (2–100 μM) were injected for 90 s followed by an additional 90-s dissociation phase. At the end of each cycle, the surface was regenerated by a 30-s injection of 1 M NaCl. The measurements were carried out in different HBSS and SIFs at 20 μL/min and at 25 °C.

Each sensorgram was determined by subtracting the nonspecific binding on the surface of the reference flow cell from the total binding on the immobilized-insulin surface. First, the equilibrium binding of each cycle was calculated using BIAevaluation software, and then the dissociation constant (KD) and the maximum amount (R_{max}) were calculated using the equilibrium amounts based on fitting by the MULTI program followed by Scatchard analysis. The maximum binding capacity under the transcellular transport assay conditions (B_{max}) was calculated using Eq. (1) as follows:

$$B_{max} = [Ins]_t \cdot R_{max}/Ins_{immob}, \quad (1)$$

where $[Ins]_t$ is the total ligand (insulin) concentration under the transcellular transport assay conditions, and Ins_{immob} is the amount of immobilized ligand (insulin). The free ($[C]_f$) and bound ($[C]_b$) concentrations of penetratin in the transcellular transport assay conditions were calculated using Eqs. (2) and (3) as follows:

$$[C]_t = [C]_f + B_{max} \times [C]_f/(KD + [C]_f), \quad (2)$$

$$[C]_b = [C]_t - [C]_f, \quad (3)$$

Table 1
Composition of SIFs used in this study.

	FaSSIF-6.5	FeSSIF-6.5	FeSSIF-5.0	NaTC (3 mM)	NaTC (15 mM)
pH	6.5	6.5	5.0	6.5	6.5
Na-TC (mM)	3	15	15	3	15
Lecithin (mM)	0.75	3.75	3.75	–	–
MES (mM)	10	10	10	10	10
MC (w/v%)	0.001	0.001	0.001	0.001	0.001
2M NaOH	q.s.	q.s.	q.s.	q.s.	q.s.
HBSS (mL)	500	500	500	500	500