Carbohydrate Polymers 87 (2012) 1822–1827

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Improving intestinal insulin absorption efficiency through coadministration of cell-penetrating peptide and hydroxypropyl-β-cyclodextrin

Liefeng Zhang[a], Lingling Song[a], Changzheng Zhang[b,*], Yong Ren[a,*]

[a] Jiangsu Key Laboratory for Supramolecular Medicinal Materials and Applications, College of Life Science, Nanjing Normal University, Nanjing 210046, China
[b] School of Life Science, Anqing Teachers College, Anhui 246011, China

ARTICLE INFO

Article history:
Received 23 June 2011
Received in revised form 29 August 2011
Accepted 3 October 2011
Available online 8 October 2011

Keywords:
Insulin
Cell-penetrating peptide
Hydroxypropyl-β-cyclodextrin
Absorption efficiency

ABSTRACT

The effects of a cell-penetrating peptide (CPP) and hydroxypropyl-β-cyclodextrin (HP-β-CD) on intestinal insulin absorption efficiency were investigated in this study. CPP (R8) and HP-β-CD were added to insulin and the *in vitro* absorption efficiency in a Caco-2 cell monolayer and *in vivo* absorption efficiency in diabetic rats were tested. The results showed that the transportation efficiency of insulin-CPP across the Caco-2 cell monolayer was 5–7 times greater and that of insulin–HP-β-CD–CPP (co-treated with HP-β-CD and CPP) was 8–10 times greater than normal insulin. Importantly, these combinations significantly reduced glycemia in diabetic rats. The formulation of insulin–HP-β-CD–CPP showed the highest increase in the permeability of insulin and the best biological response in diabetic rats of all the treatments. This study suggests that coadministration of HP-β-CD and CPP has potential in the development of oral insulin.

1. Introduction

Parenteral administration of insulin has many disadvantages such as common infections, pain due to subcutaneous injections and stress due to long-term use (Zhang et al., 2010). Delivery of insulin by non-parenteral routes has gained significant attention. Recently, substantial progress has been made in the non-invasive methods of insulin administration such as pulmonary, nasal, rectal and oral in order to replace parenteral therapy (Gomez-Perez & Rull, 2005). The oral route is recognized as the natural and safest route for drug administration. Generally, oral administration can improve disease management, enhance patient compliance and reduce the long-term complications of diabetes (Arbit, 2004). However, there are two main limitations of the oral route for insulin delivery. One is due to insulin degradation by gastrointestinal proteolytic enzymes. The other is poor permeability of insulin across intestinal epithelium due to its high molecular weight and lack of lipophilicity (Hosny, Al-Shora, & Elmazar, 2002). Therefore, it is necessary to develop a safer and more efficient oral delivery system for insulin.

Cyclodextrins (CDs) are cyclic oligosaccharides, which are recognized as useful pharmaceutical excipients (Loftson & Brewster, 1996). CDs can interact with molecules of certain size to form inclusion complexes. These inclusion complexes have been successfully used to improve the solubility, stability and bioavailability of many compounds (Peggy, Maria, & Beatriz, 2010; Zeng, Ren, Zhou, & Yu, 2011). The chemical modification of amorphous hydroxypropyl-β-cyclodextrin (HP-β-CD) by partial etherification of the crystalline parent cyclodextrin with a hydroxyalkyl group, achieves higher water solubility, greater complex properties, lower irritancy and toxicity than β-cyclodextrin (Gould & Scott, 2005; Yaksh, 1991). Our previous report showed that HP-β-CD could improve the stability of insulin in solutions containing intestinal proteases *in vitro* (Zhang et al., 2008).

Cell-penetrating peptides (CPPs) are reported to be easily taken up by cells (Futaki, 2005; Futaki et al., 2001; Mitchell, Kim, Steinman, Fathman, & Rothbard, 2000). These peptides facilitate the transduction of macromolecules such as proteins (Schwarze, Ho, Vocero-Akbani, & Dowdy, 1999), liposomes (Torchilin & Levchenko, 2003; Torchilin, Rammohan, Weissig, & Levchenko, 2001), and antibodies (Mie et al., 2003) into cells. Among several types of CPPs, the arginine-rich CPPs have been the most widely studied (El-Sayed, Futaki, & Harashima, 2009; Wender, Galliher, Goun, Jones, & Pillow, 2008). Oligoarginine, also known as cell-penetrating peptide (CPP), exerts a significant enhancing effect on intestinal insulin absorption without any untoward effects on the intestinal mucosa (Kamei, Morishita, Ehara, & Takayama, 2008; Morishita, Kamei, Ehara, Isowa, & Takayama, 2007).

In view of this, numerous strategies have been devised for the oral delivery of insulin. However, this is still one of the greatest challenges in modern pharmaceutical technology. In this study, we used HP-β-CD to overcome the enzymatic barrier, and chose an arginine-rich CPP to increase the membrane permeability of

* Corresponding authors. Tel.: +86 25 85891591; fax: +86 25 85891591.
E-mail addresses: neurobiologyzhang@yahoo.com (C. Zhang), renyong@njnu.edu.cn (Y. Ren).

0144-8617/$ – see front matter.
doi:10.1016/j.carbpol.2011.10.002

insulin. The present study aimed to identify whether CPP and HP-β-CD can improve the intestinal absorption efficiency of insulin. The ability of CPP and HP-β-CD to enhance the intestinal bioavailability of insulin was evaluated *in vitro* in a Caco-2 cell monolayer and *in vivo* in diabetic rats.

2. Materials and methods

2.1. Materials

Insulin (28 IU/mg) was purchased from Xuzhou Wangbang Biochemical Pharmaceutical Co., Ltd. (Jiangsu, China). CPP (RRRRRRRR) was synthesized by Shanghai Saijie Co., Ltd. (Shanghai, China). HP-β-CD (DS = 6.0) was purchased from Taixin Yimin Chemical Co., Ltd. (Jiangsu, China). All other chemicals were of analytical grade and are commercially available.

2.2. Determination of inclusion ratio

Absorption spectra were recorded on a Shimadzu UV-2450 spectrophotometer. Determination of the inclusion ratio was carried out using the method of gradual change in concentration (Nakajima & Hirohashi, 1984). The molar ratios of HP-β-CD and insulin were 1:1, 2:1, 4:1, 6:1, 8:1 and 10:1, respectively. The molar ratios of insulin, HP-β-CD and CPP were 1:6:0.5, 1:6:1, 1:6:2, 1:6:4, 1:6:6 and 1:6:8, respectively. These were tested in the max absorption wavelength of insulin at 276 nm. The absorption difference (ΔA) of inclusion and the corresponding insulin concentration were then calculated. A curve was constructed using the absorption difference (ΔA) as the vertical axis, and the molar ratio of the complex as the horizontal axis.

2.3. Circular dichroism

Circular dichroism (CD) was carried out according to Pourhosseini, Saboury, Najafi, and Sarbolouki (2007) with minor modifications. CD spectra were recorded at 25 °C on an Aviv 215 CD spectrophotometer (Aviv Instrument, Inc., Lake Wood, NJ, USA). Far-UV CD spectra were recorded using a 0.1-cm path length cell at 200–250 nm with a step size of 1.0 nm and a bandwidth of 1.0 nm. Aliquots of the insulin solutions were injected. Samples were incubated for 30 min prior to collecting the spectra. The background CD spectrum of a blank solution was subtracted from the sample spectra for baseline correction. Secondary structure predictions were performed using the CDNN program accessible as part of the software package of the instrument. Thermal unfolding curves were obtained in temperature mode at 222 and 217 nm by increasing the temperature from 30 to 100 °C at a heating rate of 2 °C/min. The scans were corrected for buffer contributions.

2.4. Preparation of insulin–CPP, insulin–HP-β-CD and insulin–HP-β-CD–CPP samples

2.4.1. Preparation of insulin–CPP solution

This solution was prepared according to Morishita et al. (2007) with minor modifications. Specific amounts of insulin were dissolved in 50 μL of 0.1 N HCl in polypropylene tubes, and the insulin solution was diluted with 2.0 mL of phosphate-buffered saline (PBS), pH 7.4, and then normalized with 50 μL of 0.1 N NaOH. The appropriate amount of R8 was then measured in a polypropylene tube, and an aliquot of insulin solution was added to the tubes and mixed gently to yield a clear solution. The solution contained a 1:1 molar ratio of insulin and CPP.

2.4.2. Preparation of insulin–HP-β-CD solution

Specific amounts of insulin were dissolved in 50 μL of 0.1 N HCl in polypropylene tubes, and the insulin solution was diluted with 10 mL of PBS (pH 7.4) and then normalized with 50 μL of 0.1 N NaOH. Appropriate amounts of HP-β-CD were then added. The mixture was magnetically stirred at room temperature for 2 h (this solution was called A solution). The solution contained a 1:6 molar ratio of insulin and HP-β-CD.

2.4.3. Preparation of insulin–CPP–HP-β-CD solution

A specific amount of R8 was measured in a polypropylene tube, and an aliquot of insulin–HP-β-CD solution (A solution) was added to the tubes and mixed gently to yield a clear solution. The solution contained a 1:6:1 molar ratio of insulin, HP-β-CD and CPP.

The above three solutions were then filtered through a 0.22 μm PTFE filter. The filtrates were frozen and then lyophilized for 12 h.

2.5. High performance liquid chromatography (HPLC) analysis of insulin

HPLC was carried out according to Todo, Okamoto, Iida, and Danjo (2004) with minor modifications. Analyses of the remaining insulin were carried out by reverse phase HPLC with an isocratic system (Shimadzu Co., Kyoto, Japan) using the raw insulin bulk drug as the standard (28.0 U/mg). The HPLC system was composed of a pump (LC-10ADvp), diode array detector (SPD-M10Avp), column oven (CTO-10ASvp), and LC work station (CLASS-LC10). The mobile phase was a 72:28 mixture of 0.2 M sodium sulfate buffer (pH 3.0) and acetonitrile at a flow rate of 1.0 mL/min. The column was a Shodex Asahipak ODP-506D (4.6 mm × 150 mm, 5 μm) (Showa Denko, Ltd., Tokyo) heated at 25 °C. Ultraviolet absorption was measured at 214 nm.

2.6. Degradation of insulin induced by α-chymotrypsin and trypsin

Specific amounts of insulin were dissolved in 50 μL of 0.1 N HCl in polypropylene tubes, and the insulin solution was diluted with 2 mL of PBS (pH 7.4), and then normalized with 50 μL of 0.1 N NaOH. α-Chymotrypsin (5 mg/mL) and trypsin (12 mg/mL) solutions were prepared with Tris–HCl buffer (pH = 8.0), respectively. Different treatments were prepared. The final concentration of insulin in the solution was 0.5 mg/mL. Before the addition of α-chymotrypsin solution, the insulin solution was vortexed at high speed for 5 s and a sample was withdrawn and added to ice-cold 0.5% trifluoroacetic acid (TFA) to obtain the zero time sample. Samples were withdrawn at 15, 30, 45 and 60 min after the addition of the enzyme solution at 37 °C. The final enzyme concentration of α-chymotrypsin and trypsin were 8 mg/L (Tang, Yin, Pei, Yu, & Cui, 2004) and 600 mg/L (Qi, Ping, Xu, & Shi, 2004), respectively. The samples were then analyzed by HPLC after adding 50 μL of ice-cold 0.5% TFA to terminate the reaction. Each experiment was carried out three times. The average and standard deviation of the data were calculated.

2.7. In vitro intestinal absorption assay in a cultured Caco-2 cell monolayer

Caco-2 cell monolayers were prepared by cultivating Caco-2 cells on polycarbonate membrane filters for 3 weeks (Degim, Unal, & Abbasoglu, 2001). The Caco-2 cell monolayer on the membrane filter was placed between donor and receptor compartments acting as a membrane. Cells were incubated at 37 °C and samples were added to the donor chamber, and the receptor compartment was filled. Concentrations of insulin in these samples were analyzed by HPLC. The effective permeability (P_{eff}) was calculated based on

the appearance of insulin in the receiver chamber under sink conditions: $P_{eff}\ 1/4 = J_{ss}/C_D = (dC_R \cdot V_R/d_t \cdot A)\cdot(1/C_D)$, where dC_R/d_t is the change in insulin concentration in the receiver chamber at steady-state, V_R is the volume of receiver buffer, A is the cross-sectional area of the exposed tissue, and C_D is the drug concentration in the donor chamber.

2.8. Oral administration of insulin

Male Wistar rats (250–300 g, 1–2 months old, blood glucose levels > 16 mmol/L) were fasted overnight, but had free access to water. The diabetic rats were divided into five groups each containing a minimum of 5 rats. The first four groups received either insulin or insulin–CD or insulin–CPP or insulin–CD–CPP formulations at a dose of 50 IU/kg animal body weight. The fifth group was the diabetic control. The samples were placed in enteric-coated capsules (purchased from Qiangji Pharmaceutical Factory, China, exclusively for small animals), and then administered orally. Blood glucose was determined by the glucose oxidase method (glucose determination agent box, Shanghai Rongsheng Biological Technology Co., Ltd., China) (Wu, Ping, Lai, & Wei, 2003). All the experimental treatments were in agreement with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

2.9. Statistical analysis

Each result shown in the figures is the mean of at least three repeated treatments. The significance of the differences between treatments was evaluated statistically by standard deviation and Student's *t*-test methods, and a *P* value of <0.05 was considered of statistical significant.

3. Results

3.1. Determination of inclusion ratio

The gradual change in concentration is the common usual method used to determine the inclusion ratio (Nakajima & Hirohashi, 1984). According to Fig. 1A, the value of ΔA first increased when the ratio of HP-β-CD and insulin increased. However, when the ratio was greater than 6:1, the value of ΔA was almost constant. This suggested that the amount of HP-β-CD was excessive. Therefore, a ratio of HP-β-CD and insulin of 6:1 was used in this study.

CPP is known to facilitate the transduction of macromolecules. In this study, CPP was added to the complex of HP-β-CD and insulin. From Fig. 1B, the value of ΔA was constant, and then decreased when CPP increased. It showed that CPP did not affect the complex when amount of CPP was low. However, excessive CPP weakened inclusion of HP-β-CD and insulin. CPP may compete during the formation of the complex between HP-β-CD and insulin, since CPP is smaller than insulin. However, this requires further study to explain the mechanism. In this study, the ratio of insulin, HP-β-CD and CPP was 1:6:1. This ratio did not affect the complex.

3.2. Circular dichroism spectroscopy

Far-UV CD spectra are usually used as a fingerprint for the identification of various secondary structural elements (Ahmad, Uversky, Hong, & Fink, 2005). Far-UV CD spectra of insulin in the presence of various formulations are shown in Fig. 2. Native insulin, without formulations, exhibited the typical spectrum of an α-helical protein, with the characteristic minima at 208 and 222 nm. No significant difference was found between the native insulin and insulin–CPP. However, changes in the secondary structure of insulin in the presence of HP-β-CD were reflected in the intensity

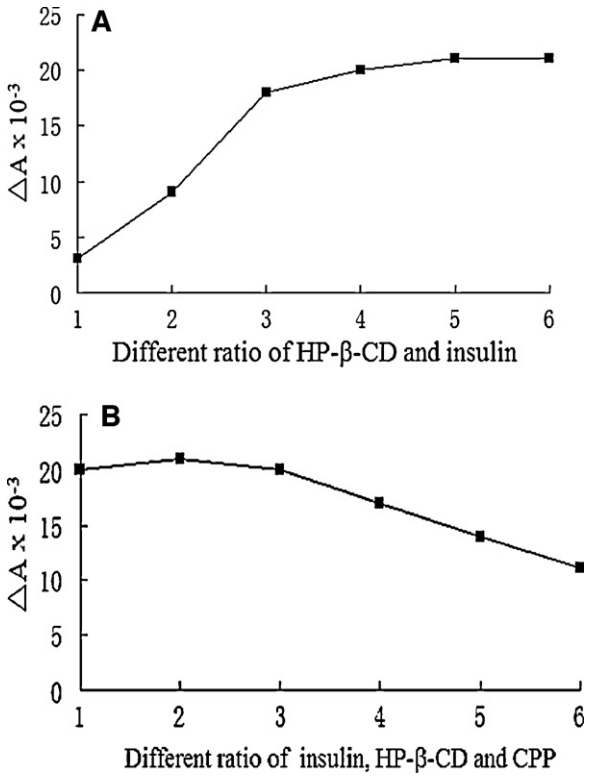

Fig. 1. Gradual change of insulin, HP-β-CD and CPP.

of the spectra in the vicinity of the two wavelengths described. The α-helical content of insulin exhibited a small rise. Fig. 2 also showed that the CD spectra of insulin–HP-β-CD and insulin–HP-β-CD–CPP partly overlapped, indicating that insulin in both solutions had a very similar secondary structure. Altogether, these results indicated that HP-β-CD changed the secondary structure of insulin, to some extent, and CPP had no effect on the secondary structure of insulin. This suggested that CPP and insulin were just mixed, but insulin and HP-β-CD formed a complex. In addition, researchers

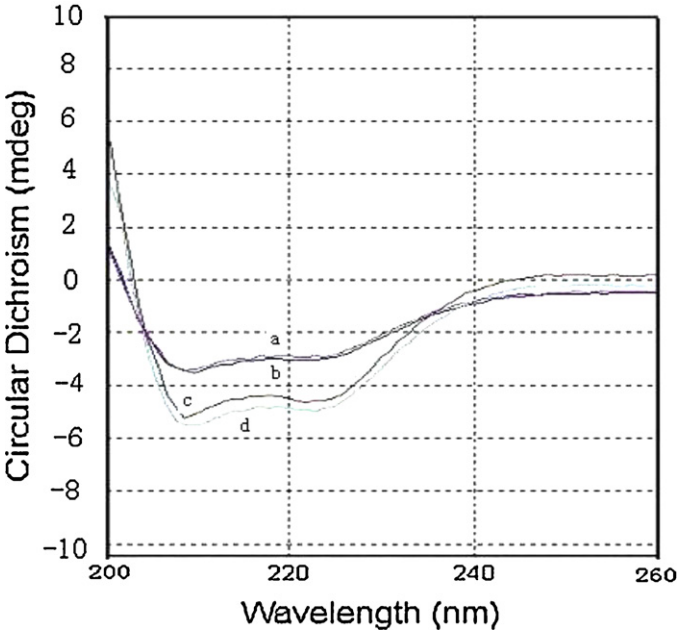

Fig. 2. Far-UV CD spectra.

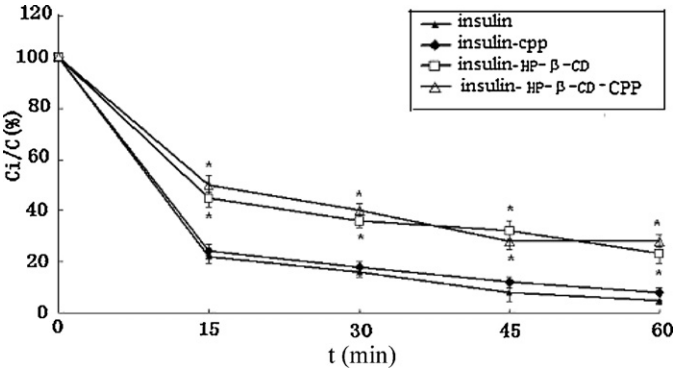

Fig. 3. Percentage of insulin remaining in the presence of α-chymotrypsin at 37 °C.

have also identified the formation of a complex between insulin and cyclodextrin using various methods (Dotsikas & Loukas, 2002; Sajeesh & Sharma, 2006). These findings were consistent with those in the current study.

3.3. Effects of HP-β-CD and CPP on degradation of insulin induced by α-chymotrypsin and trypsin in vitro

α-Chymotrypsin is a common intestinal protease. The degradation of insulin by α-chymotrypsin was investigated *in vitro*. The remaining insulin was measured by HPLC (Fig. 3). Insulin was degraded rapidly by α-chymotrypsin, and most of the insulin was degraded within 60 min in the control and insulin–CPP formulation. However, about 23% of insulin was detected in the presence of HP-β-CD, and about 28% of insulin was detected in the presence of HP-β-CD and CPP. These results demonstrated that there were no significant differences between insulin and insulin–CPP, or between insulin–HP-β-CD and insulin–HP-β-CD–CPP. These findings suggested that CPP had little effect on protecting insulin from degradation by α-chymotrypsin. However, following statistical analysis, it was found that the effects of HP-β-CD had statistical significance ($P < 0.05$). HP-β-CD had an advantage on the protection of insulin.

To further characterize degradation of insulin by the intestinal enzyme, insulin was incubated in the presence of trypsin, another common intestinal protease. These results are shown in Fig. 4. The amount of insulin decreased with incubation time from 0 to 60 min, and only about 15% of insulin remained after 60 min. The degradation of insulin was partly inhibited in the presence of HP-β-CD, and was significantly decreased when incubated in the presence of

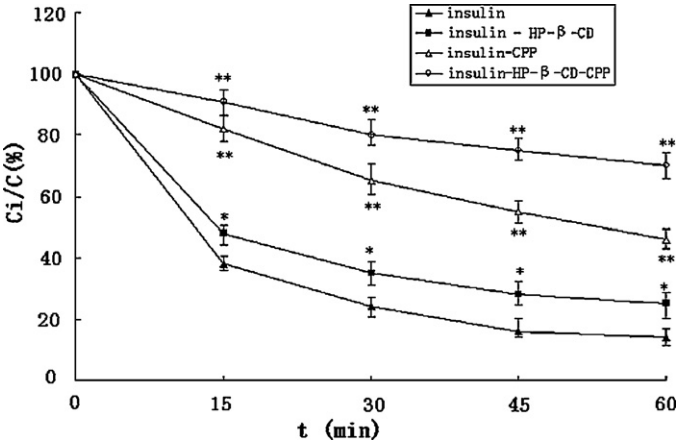

Fig. 4. Amount of insulin transported across Caco-2 cell monolayer during the permeation experiments ($n = 3$).

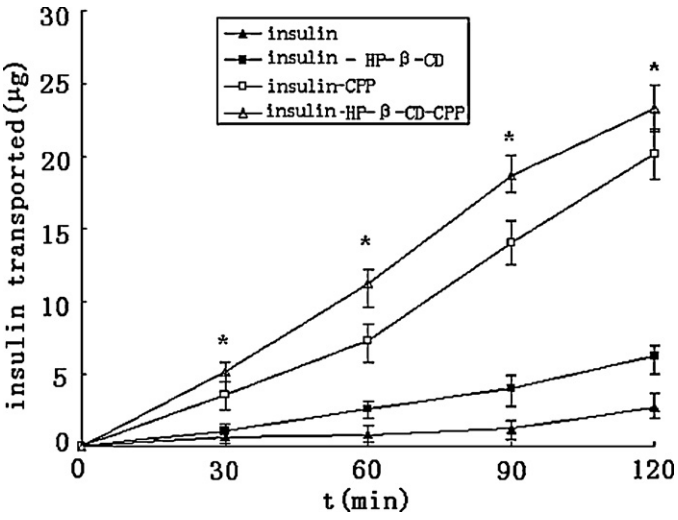

Fig. 5. Percentage of insulin remaining in the presence of trypsin at 37 °C.

CPP. When insulin was co-incubated with HP-β-CD and CPP, about 70% of insulin was detected at 60 min ($P < 0.01$). This suggested that HP-β-CD and CPP had synergistic effects on protecting insulin from degradation by trypsin (Fig. 5).

Taken together, these results showed that CPP (R8) had little effect on protecting insulin from degradation by α-chymotrypsin, but was useful in resisting degradation by trypsin. Trypsin cleaved insulin at the B29-Lys and B22-Arg residues of insulin. The B22-Arg residue is essential to achieve greater stability without losing activity (Schilling & Mitra, 1991). The amino acid residues of CPP (R8) are all Arg residues. Therefore, CPP (R8) may protect insulin from degradation by the mechanism of competition and combination with proteolytic enzymes. α-Chymotrypsin appeared to cleave initially at the carboxyl side of the B26-Tyr and A19-Tyr residues. In addition, cleavage at B16-Tyr, B25-Phe, and A14-Tyr residues also occurred rapidly (Qi et al., 2004; Schilling & Mitra, 1991). CPP had not the same amino residues as the cleavage site by α-chymotrypsin. Thus, CPP had no effect on the degradation of insulin by α-chymotrypsin. In conclusion, HP-β-CD and CPP both protected insulin from degradation by intestinal proteases.

3.4. Intestinal absorption assay using the in vitro Caco-2 cell monolayer model

The transport properties of insulin, insulin–HP-β-CD, insulin–CPP and insulin–HP-β-CD–CPP were compared *in vitro* in a Transwell-grown Caco-2 cell monolayer, a widely used *in vitro* model for the study of intestinal absorption. Caco-2 cells, which are derived from a human colorectal carcinoma, form a highly polarized membrane when grown to confluence on microporous filters and show close morphological and functional similarities with intestinal epithelium (Anderle et al., 1998). These experiments were performed to investigate the effects of CPP and HP-β-CD on the transport properties of insulin across the Caco-2 cell monolayer. As shown in Fig. 4, the transportation efficacy of insulin crossing the Caco-2 cell monolayer was extremely low, however, HP-β-CD, to some extent, caused an increase in the transport of insulin across the monolayer. The presence of CPP significantly enhanced the transportation efficiency. These results are in agreement with previous reports, where cyclodextrin increased the absorption of insulin (Sajeesh & Sharma, 2006; Shao, Krishnamoorthy, & Mitra, 1992). Morishita et al. (2007) reported that insulin absorption increased dramatically following coadministration of R8 in a dose-dependent manner.

Table 1
Comparison of the effective permeability of insulin.

Formulation	$P_{epp} \times 10^{-5}$
Control	0.552 ± 0.023
Insulin + CD	0.925 ± 0.035
Insulin + CPP	3.668 ± 0.086
Insulin + CD + CPP	5.024 ± 0.097

Higher transportation efficiency was obtained with the insulin–HP-β-CD–CPP formulation in this study. Transport across the Caco-2 cell monolayer was in the order: insulin–HP-β-CD–CPP > insulin–CPP > insulin–HP-β-CD > insulin, with a 9-, 7-, and 2-fold increase compared to native insulin as calculated by effective permeability (P_{epp} value) (Table 1). To date, synergic effects of HP-β-CD and CPP on the transport of insulin across the monolayer have not been reported. In this study, we found that HP-β-CD–CPP showed the highest increase in the permeability of insulin across the Caco-2 cell monolayer of all the formulations studied.

3.5. Insulin delivery in diabetic rats

The above results showed that CPP and HP-β-CD could improve the intestinal absorbance of insulin. Further research including the actual intestinal absorption of insulin in diabetic animals is underway.

The impacts of the novel formulations on glucose level in diabetic rats are summarized in Fig. 6. These findings show the variation in glycemia in diabetic rats expressed as a percentage of the animal's initial glycemia after oral administration of either an insulin-containing formulation or control. No reduction in glycemia was observed in the control group. This demonstrated the stable diabetic level in the experimental animals. When a single dose of insulin (50 IU/kg) was administered, a hypoglycemic effect was not observed. A reduction in glycemia was observed following treatment with HP-β-CD. From the statistical analysis, this finding had no statistical significance ($P > 0.05$). However, a marked reduction in glycemia was also observed with insulin–CPP and insulin–HP-β-CD–CPP. After 2 h, the insulin–CPP formulation induced a reduction of 19% in the initial value of glycemia. The insulin–HP-β-CD–CPP formulation induced a 35% reduction. The blood glucose level was maintained at a low level for up to 4 h. The maximum hypoglycemic effect was observed with the insulin–HP-β-CD–CPP formulation.

The CPP significantly reduced glycemia in diabetic rats. CPP is a useful tool for delivering macromolecules across cell membranes, which has been reported by various researchers (Kamei et al., 2008; Morishita et al., 2007). The results obtained in this study were in agreement with those reported in these previous publications. However, our study used dose of CPP which is significantly lower than previous reported. Furthermore, the novel formulation of insulin–HP-β-CD–CPP could dramatically reduce glycemia in diabetic rats, which has not been reported before. The coadministration of HP-β-CD and CPP improved the efficiency of insulin absorption. The efficiency of coadministration of HP-β-CD and CPP was better than each alone. Some strategies have been proposed to boost oral insulin bioavailability. These strategies include enzyme inhibitors (Yamamoto et al., 1994) and encapsulation technologies such as nanoparticles (Krauland & Alonso, 2007). However, these strategies require relatively high insulin doses. Some promising oral delivery techniques in the range of 75–100 IU/kg have also been reported (Fasano & Uzzau, 1997). In this study, the doses of insulin and CPP were both lower than those in previous publications. Low insulin bioavailability has been one of the main hurdles in bringing oral insulin delivery into clinical practice (Whitehead, Shen, & Mitragotri, 2004). In addition, we speculate that this novel formulation of insulin–HP-β-CD–CPP combined with new methods in pharmacy could further improve oral insulin bioavailability. The further research is under progress in our laboratory.

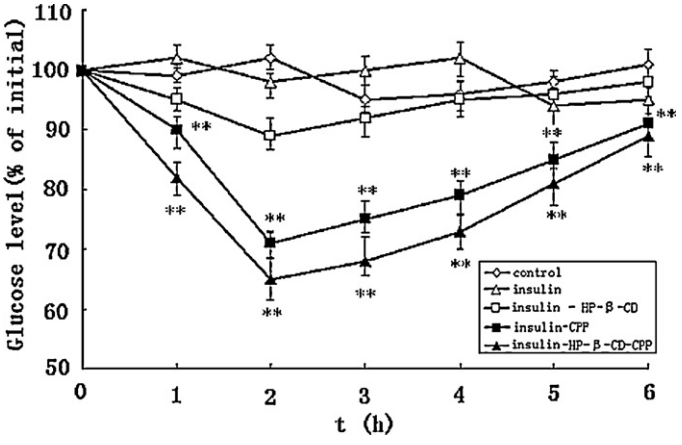

Fig. 6. Plasma glucose levels after oral administration in diabetic rats.

4. Conclusions

In this study, an oral insulin delivery system consisting of CPP and HP-β-CD was evaluated. We found that HP-β-CD and CPP protected insulin from degradation by intestinal proteases *in vitro*. CPP and HP-β-CD also enhanced insulin transport across the Caco-2 cell monolayer, and a synergic effect was also observed. Taken together, the formulation of insulin–HP-β-CD–CPP induced the greatest increase in the permeability of insulin and resulted in the best biological response in diabetic rats of the formulations studied.

Altogether, these observations demonstrate that coadministration of HP-β-CD and oligoarginine (R8) can markedly increase intestinal insulin absorption. These results may provide some guidance for the future development of the oral formulation of insulin, which is currently under active investigation in our laboratory.

Acknowledgments

This study was financially supported by the Postdoctoral Special Foundation of China (No. 200902524) and Natural Science Foundation of Jiangsu Higher Education Institutions (No. 09KJD18000).

References

Ahmad, A., Uversky, V. N., Hong, D. & Fink, A. L. (2005). Early events in the fibrillation of monomeric insulin. *The Journal of Biological Chemistry*, *280*, 42669–42675.
Anderle, P., Niederer, E., Rubas, W., Hilgendorf, C., Spahn-Langguth, H., Wunderli-Allenspach, H., et al. (1998). P-Glycoprotein (P-gp) mediated efflux in Caco-2 cell monolayers: the influence of culturing conditions and drug exposure on P-gp expression levels. *Journal of Pharmaceutical Sciences*, *87*, 757–762.
Arbit, E. (2004). The physiological rationale for oral insulin administration. *Diabetes Technology & Therapeutics*, *6*, 510–517.
Degim, Z., Unal, N. & Abbasoglu, U. (2001). Caco-2 cell culture as a model for famotidine and insulin absorption across the intestinal mucosa. In *World Congress of Pharmacy and Pharmaceutical Sciences 61st International Congress of FIP* Singapore,. Abstracts, p. 53.
Dotsikas, Y. & Loukas, Y. L. (2002). Kinetic degradation study of insulin complexed with methyl-beta cyclodextrin. Confirmation of complexation with electrospray mass spectrometry and 1H NMR. *Journal of Pharmaceutical and Biomedical Analysis*, *29*, 487–494.
El-Sayed, A., Futaki, S. & Harashima, H. (2009). Delivery of macromolecules using arginine-rich cell-penetrating peptides: Ways to overcome endosomal entrapment. *The AAPS Journal*, *11*, 13–22.
Fasano, A. & Uzzau, S. (1997). Modulation of intestinal tight junctions by zonula occludens toxin permits enteral administration of insulin and other macromolecules in an animal model. *Journal of Clinical Investigation*, *99*, 1158–1164.
Futaki, S. (2005). Membrane-permeable arginine-rich peptides and the translocation mechanisms. *Advanced Drug Delivery Reviews*, *57*, 547–558.
Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., et al. (2001). Arginine-rich peptides. An abundant source of membrane-permeable peptides

having potential as carriers for intracellular protein delivery. *The Journal of Biological Chemistry*, *276*, 5836–5840.
Gomez-Perez, F. J. & Rull, J. A. (2005). Insulin therapy: Current alternatives. *Archives of Medical Research*, *36*, 258–272.
Gould, S. & Scott, R. C. (2005). 2-Hydroxypropyl beta cyclodextrin: A toxicological review. *Food and Chemical Toxicology*, *43*, 1451–1459.
Hosny, E. A., Al-Shora, H. I. & Elmazar, M. M. A. (2002). Oral delivery of insulin from enteric-coated capsules containing sodium salicylate: effect on relative hypoglycemia of diabetic beagle dogs. *International Journal of Pharmaceutics*, *26*, 71–76.
Kamei, N., Morishita, M., Ehara, J. & Takayama, K. (2008). Permeation characteristics of oligoarginine through intestinal epithelium and its usefulness for intestinal peptide drug delivery. *Journal of Controlled Release*, *131*, 94–99.
Krauland, A. H. & Alonso, M. J. (2007). Chitosan/cyclodextrin nanoparticles as macromolecular drug delivery system. *Pharmaceutical Nanotechnology*, *340*, 134–142.
Loftson, T. & Brewster, M. E. (1996). Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. *Journal of Pharmaceutical Science*, *85*, 1017–1025.
Mie, M., Takahashi, F., Funabashi, H., Yanagiba, Y., Aizawa, M. & Kobatake, E. (2003). Intracellular delivery of antibodies using TAT fusion protein A. *Biochemical and Biophysical Research Communications*, *310*, 730–734.
Mitchell, D. J., Kim, D. T., Steinman, L., Fathman, C. G. & Rothbard, J. B. (2000). Polyarginine enters cells more efficiently than other polycationic homopolymers. *The Journal of Peptide Research*, *56*, 318–325.
Morishita, M., Kamei, N., Ehara, J., Isowa, K. & Takayama, K. (2007). A novel approach using functional peptides for efficient intestinal absorption. *Journal of Controlled Release*, *118*, 177–184.
Nakajima, T. & Hirohashi, M. (1984). Studies of cyclodextrin inclusion complexes. 1. Complex between cyclodextrins and bencylane in aqueous solution. *Chemical & Pharmaceutical Bulletin*, *32*, 383.
Peggy, A. P., Maria, P. B. & Beatriz, E. E. (2010). Encapsulation of cinnamon and thyme essential oils components (cinnamaldehyde and thymol) in β-cyclodextrin: Effect of interactions with water on complex stability. *Journal of Food Engineering*, *99*, 70–75.
Pourhosseini, P. S., Saboury, A. A., Najafi, F. & Sarbolouki, M. N. (2007). Interaction of insulin with a triblock copolymer of PEG-(fumaric-sebacic acids)-PEG: Thermodynamic and spectroscopic studies. *Biochimica et Biophysica Acta*, *1774*, 1274–1280.
Qi, R., Ping, Q. N., Xu, R. Y. & Shi, Y. P. (2004). Effect of casein and protamine on the enzymatic degradation and the orally hypoglycemic action of insulin. *Acta Pharmaceut Sinica*, *39*, 844–848.
Sajeesh, S. & Sharma, C. P. (2006). Cyclodextrin–insulin complex encapsulated polymethacrylic acid based nanoparticles for oral insulin delivery. *International Journal of Pharmaceutics*, *325*, 147–154.
Schilling, R. J. & Mitra, A. K. (1991). Degradation of insulin by trypsin and alpha-chymotrypsin. *Pharmaceutical Research*, *8*, 721–727.
Schwarze, S. R., Ho, A., Vocero-Akbani, A. & Dowdy, S. F. (1999). In vivo protein transduction: Delivery of a biologically active protein into the mouse. *Science*, *285*, 1569–1572.
Shao, Z., Krishnamoorthy, R. & Mitra, A. K. (1992). Cyclodextrins as nasal absorption promoters of insulin: mechanistic evaluations. *Pharmaceutical Research*, *9*, 1157–1163.
Tang, C., Yin, C. H., Pei, Y. Y., Yu, L. & Cui, F. Y. (2004). Study on the stability of oral insulin in gastrointestinal tract. *Chinese Pharmaceutical Journal*, *39*, 764–766.
Torchilin, V. P. & Levchenko, T. S. (2003). TAT-liposomes: A novel intracellular drug carrier. *Current Protein & Peptide Science*, *4*, 133–140.
Torchilin, V. P., Rammohan, R., Weissig, V. & Levchenko, T. S. (2001). TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitor. *Proceedings of the National Academy of Sciences*, *98*, 8786–8791.
Todo, H., Okamoto, H., Iida, K. & Danjo, K. (2004). Improvement of stability and absorbability of dry insulin powder for inhalation by powder-combination technique. *International Journal of Pharmaceutics*, *271*, 41–52.
Wender, P. A., Galliher, W. C., Goun, E. L., Jones, L. R. & Pillow, T. H. (2008). The design of guanidinium-rich transporters and their internalization mechanisms. *Advanced Drug Delivery Reviews*, *60*, 452–472.
Whitehead, K., Shen, Z. & Mitragotri, S. (2004). Oral delivery of macromolecules using intestinal patches: Applications for insulin delivery. *Journal of Controlled Release*, *98*, 37–45.
Wu, Z. H., Ping, Q. N., Lai, J. M. & Wei, Y. (2003). Hypoglycemic effect of polysaccharide-coated insulin liposomes after oral administration in mice. *Acta Pharmacologica Sinica*, *38*, 138–142.
Yaksh, T. L. (1991). The Utility of 2-hydroxypropyl beta cyclodextrin as a vehicle for the intracerebral and intrathecal administration of drugs. *Life Sciences*, *48*, 623–634.
Yamamoto, A., Taniguchi, T., Rikyuu, K., Tsuji, T., Fujita, T., Murakami, M., et al. (1994). Effects of various protease inhibitors on the intestinal absorption and degradation of insulin in rats. *Pharmaceutical Research*, *11*, 1496–1500.
Zeng, J., Ren, Y., Zhou, C. & Yu, S. (2011). Preparation and physicochemical characteristics of the complex of edaravone with hydroxypropyl-β-cyclodextrin. *Carbohydrate Polymers*, *83*, 1101–1105.
Zhang, L., Jiang, H., Zhu, W., Wu, L., Song, L., Wu, Q. Y., et al. (2008). Improving the stability of insulin in solutions containing intestinal proteases *in vitro*. *International Journal of Molecular Sciences*, *9*, 2376–2387.
Zhang, N., Li, J., Jiang, W., Ren, C., Li, J., Xin, J., et al. (2010). Effective protection and controlled release of insulin by cationic β-cyclodextrin polymers from alginate/chitosan nanoparticles. *International Journal of Pharmaceutics*, *393*, 212–218.