A Potential Mechanism of a Cationic Cyclopeptide for Enhancing Insulin Delivery across Caco-2 Cell Monolayers

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Received June 17, 2013; accepted August 3, 2013; advance publication released online August 8, 2013

Effective delivery of therapeutic biomolecules across biomembranes is a challenging topic. A cationic cyclopeptide named TD-34 (ACSSKKSKHCG) was reported to improve insulin delivery across biomembranes effectively. Based on our previous work, we investigated the mechanism of TD-34 for enhancing insulin delivery across Caco-2 cell monolayers. Transport studies of insulin, TD-34 and insulin accompanied with TD-34 were performed respectively using Caco-2 cell monolayers at different conditions. Transepithelial electrical resistance (TEER) value was monitored for 24 h immediately after the beginning of transport experiments. Moreover, the tight junction protein (Claudin-1) was localized by confocal immunofluorescence microscopy. Results showed the transport of insulin alone across biomembranes was attributable to multiple routes including passive diffusion. When TD-34 accompanied with or without insulin was treated on Caco-2 cell monolayers, TEER values decreased reversibly, and it was correlated with the reappearance of tight junction proteins by immunostaining assay. It was concluded that the cationic cyclopeptide (TD-34) had the potential to enhance paracellular delivery of insulin across Caco-2 cell monolayers by loosening tight junction reversibly.

Key words insulin; cationic cyclopeptide; tight junction; endocytosis; paracellular delivery

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tion (U.S.A.) and Boster (Beijing, China), respectively. Caco-2 cells were acquired from Institute of Cell Bank (Shanghai, China). All other reagents were of analytical or chromatographic grade.

**Synthesis of the Peptide** TD-34 was prepared by standard solid phase peptide synthesis method according to Fmoc strategy using microwave technology (CEM Corporation, U.S.A.). 2-((1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) were used as the coupling reagent, and Wang-resin as the solid support. The amino acids were 3-fold in excess with the coupling progress carried out at 60°C using 20 W microwave power for 5 min. Fmoc was deprotected with 20% (v/v) piperidine in N,N-dimethylformamide (DMF) with the deprotection progress carried out at 60°C using 20 W microwave power for 3 min. To obtain linear TD-34, trifluoroacetic acid (TFA), water and TIS (95:2.5:2.5, v/v/v) were used as cleavage solution for 2 h at room temperature. In order to prepare cyclopeptide, linear TD-34 was precipitated by ice diethyl ether, and then TD-34 dropwisely added to 25% (v/v) methanol contained 2.5 eq. iodine with stirring at room temperature for the disulfide bonds reaction. At last, TD-34 was purified by Sephadex G-15 chromatography using 50% (v/v) methanol. The purity (> 96%) was analyzed by HPLC (LC2010A, Japan) and molecular weight ([MW + H]+=1133.6, Cal. MW=1132.5) was analyzed by electrospray ionization-mass spectrum (ESI-MS) (Agilent 6224, U.S.A.), respectively.

**Preparation of the Transport Solution** Insulin was dissolved in 50 µL 0.01 mol/L HCl, and then diluted with Hank’s buffered saline solution (HBSS) before adjusted to about pH=7.0 with 0.01 mol/L NaOH. The insulin solution (pH=7.0) was finally blended with TD-34.

**Stability of TD-34 in HBSS** The stability of TD-34 during the experiment was determined in HBSS. Five micromoles per milliliter TD-34 was incubated in a controlled water bath at 37°C. Samples were withdrawn from the solution at predetermined time points (0, 1, 2, 8 h). All measurements were performed in triplicate and the amount of the peptide was quantified by HPLC.

**Cell Culture** Caco-2 cells (passage 40–60) were grown in 25 cm² flasks and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% (v/v) fetal bovine serum, 1% nonessential amino acids, 2mmol/L L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin (pH=7.4) in an incubator at 37°C with 5% CO₂, 95% air and 90% relative humidity. The cells were given fresh medium three times a week.

**Cytotoxicity Assays** The effect of synthesized peptides containing insulin on cell viability was measured using MTT assay at the concentration of 5 µmol/mL TD-34 and 21 IU/mL insulin. Caco-2 cells (2×10⁴ cells/mL) were seeded in 96-well tissue culture plates for 24 h. The cells were incubated with 200 µL solution (HBSS alone as a control group and the other group is HBSS containing TD-34 and insulin) for 24 h. Thereafter, 500 µg/mL MTT was added to each well and cells were incubated for another 4 h. Medium was then replaced with 200 µL dimethyl sulfoxide (DMSO) and measured at 550nm using a microplate reader (SpectraMax Plus 384, U.S.A.). Percentage of cell viability was calculated and compared to the control.

**Transport of Insulin or TD-34** Caco-2 cells at a density of approximately 2×10⁴ cells/mL were seeded in 12-well plates (3460 Transwell, Costar, U.S.A.). Caco-2 cells were harvested after seeding 20–22 d for relevant experiments. After removal of the culture medium, each well was washed and preincubated with HBSS containing 25 mmol/L N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) (pH =7.4) for 10 min at 37°C or 4°C. TEER of all the wells at 37°C over 400 Ω · cm² were used for further experiment to ensure the integrity of the monolayers (Millicell® ERS-2, Millipore, U.S.A.). The transport medium containing 21 IU/mL insulin or 5 µmol/mL TD-34 was added to the apical (0.5 mL) or basolateral (1.5 mL) compartment; when 5 µmol/mL TD-34 was accompanied with 21 IU/mL insulin, the transport medium was added to the basolateral (1.5 mL) compartment. The receiving compartment was added to 0.5 mL transport medium. All of the transport experiments were performed at 37°C or 4°C, respectively. One hundred microliter sample solution was withdrawn from the receiver at 0.5, 1, 1.5 and 2 h, and was replenished with an equal volume of pre-warmed or pre-cooled transport medium immediately. After transport experiment, transport medium was replaced by fresh culture medium. TEER value was measured for 24 h at predetermined time points immediately after the beginning of transport experiments. The concentrations of insulin in the samples were determined by reversed phase HPLC with reference to a calibration curve.

**Transport of Insulin or TD-34 with Endocytosis Inhibitor** A stock solution of phenylarsine oxide (PAO) was prepared in DMSO at the concentration of 25 µmol/L. Solutions containing insulin or TD-34 was prepared immediately before the beginning of transport experiments. The final concentration of DMSO in transport medium was less than 0.5%. To study the effect of endocytosis inhibitor, cells were incubated in the presence of PAO for 10 min. Thereafter, cells were washed three times and incubated in 21 IU/mL insulin for 2 h with or without PAO, and 100 µL sample was withdrew at predetermined time, the other procedure was the same as “Transport of insulin or TD-34.”

**Immunofluorescence Microscopy** Caco-2 cells were seeded on cover glasses in 24-well plates at a density of 2×10⁴ cells per well and cultured for 5 d. After the medium was removed, the cells were washed twice with prewarmed HBSS, and then incubated with HBSS at 37°C for 30 min. After HBSS was removed, the cells were incubated in three groups. DMEM was added in group I as control, and 5 µmol/mL TD-34 was added in group II and group III. After 2 h incubation, the medium was removed and cells were washed three times for 5 min each in phosphate buffered saline (PBS) to remove residual peptide. The cells of group I and group II were fixed with freshly prepared 95% (v/v) ethanol at 4°C overnight, and group III was incubated in DMEM for another 22 h before fixed. Fixed cells were treated with PBS containing 0.5% (v/v) Triton-100 followed by twice washes with PBS for 5 min each. Non-specific bindings were blocked with PBS containing 1% bovine serum albumin (BSA) for 1 h. After three washes using PBS, the cells were incubated with 2.5 µg/mL Rabbit anti-Claudin-1 overnight at 4°C, then washed with PBS three times for 5 min each and stained with 10 µg/mL CY3 goat anti-rabbit secondary antibody for 1 h at 37°C. Each specimen was washed three times in PBS and imaged using confocal laser scanning microscope (Olympus FV100, U.S.A.).
Data and Statistical Analysis  HPLC analysis method of insulin and TD-34 was the same as our previous study.\(^3\)

\[ P_{app} = \frac{dQ/dt}{A - C_0} \]  
\(dQ/dt\) is the flux rate (\(\mu\)mol/s). \(A\) is the effective surface area of the cell monolayers (1.1 cm\(^2\)). \(C_0\) is the initial concentration of insulin or TD-34 on the donor side (\(\mu\)mol/mL).

Permeability direction ratio (PDR) was calculated according to Eq. 2:

\[ PDR = \frac{P_{app(AP-BL)}}{P_{app(BL-AP)}} \]  

where AP–BL is the apical to basolateral transport and BL–AP is the basolateral to apical transport.

All experiments were performed in at least three parallel groups. Transportation data was expressed as mean±S.E.M. and analyzed by Student’s \(t\)-test to determine the statistical significant differences between two groups. One-way analysis of variance (ANOVA) followed by Dunnett’s test was used to determine the statistical significance in comparisons of more than two groups. *\(p<0.05\) and **\(p<0.01\) were considered as statistically significant in the figures and tables of this manuscript.

RESULTS AND DISCUSSION

Stability of TD-34 in HBSS  The concentration of TD-34 has been maintained the same in HBSS for 8 h at least (Fig. 1). This result indicated the TD-34 was stable during the experiment and the analysis. Therefore, TD-34 solution was freshly prepared just before the transport experiment.

Cytotoxicity of TD-34 Accompanied with Insulin  Previous study manifested that cytotoxicity of 5 \(\mu\)mol/mL TD-34 alone was not detected on Caco-2 cells, and the viability of cells was 111.7 ±20%. The cytotoxicity of 5 \(\mu\)mol/mL TD-34 accompanied with 21 IU/mL insulin was further evaluated using MTT assay. The viability of Caco-2 cells was 98 ±13%. The experiment result was well consistent with the result in our previous experiments for MTT assay. No significant cytotoxicity of 5 \(\mu\)mol/mL TD-34 was observed with or without 21 IU/mL insulin. The following experiments were done at this concentration.

Transport of Insulin or TD-34 across Caco-2 Cell Monolayers  To determine the potential route of insulin or TD-34 delivery across Caco-2 cell monolayers, transport flux of insulin or TD-34 was measured during the transport experiments and \(P_{app}\) value was calculated. The bidirectional transport results of 21 IU/mL insulin and 5 \(\mu\)mol/mL TD-34 were summarized in Table 1. The absorptive \(P_{app}\) values (AP–BL) of insulin and TD-34 were both larger than their secretory \(P_{app}\) values (BL–AP). PDR value which was higher than 1.5 indicated the transport was an active process.\(^2\) Based on the above result, it could be inferred that insulin and TD-34 transported across Caco-2 cell monolayers mainly by an active process.

To further investigate transport route of insulin and TD-34, effect of endocytosis inhibitor on transport of insulin and TD-34 was tested. After Caco-2 cell monolayers were treated with 25 \(\mu\)mol/L PAO, \(P_{app}\) value (AP–BL) of insulin decreased to 1.10×10\(^{-6}\) cm/s, but \(P_{app}\) value (BL–AP) in the opposite direction was nearly unchanged (Table 1), this result demonstrated transport of insulin across Caco-2 cell monolayers (AP–BL) was primarily attributable to the transcellular delivery, an endocytosis process. But endocytosis route was not the main route when insulin transported across secretory side of Caco-2 cell monolayers. On the other hand, \(P_{app}\) value of TD-34 reduced in both directions after cells were treated with PAO, it indicated TD-34 transported across Caco-2 cell monolayers mainly by an endocytosis.

Effect of Temperature on Transport of Insulin and TD-34  The effect of temperature on transport of insulin and TD-34 across Caco-2 cell monolayers was determined by measuring \(P_{app}\) value at 4°C. The transport of insulin was not completely eliminated at 4°C, but in AP–BL and BL–AP direction \(P_{app}\) values reduced to 5.54% and 40.74% compared to 37°C, respectively (Table 1). Furthermore, PDR of insulin at 4°C decreased to 1.64, indicating an involvement of a temperature dependent process. As the transport of insulin was not

**Table 1. Permeability of Insulin or TD-34 in Caco-2 Cell Monolayers (\(n=3\))**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Insulin(^a)</th>
<th>TD-34(^b)</th>
<th>PAO(^c)</th>
<th>(P_{app}(\times10^{-6}, \text{cm/s}))</th>
<th>Ratio PDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>(3.25\pm0.23) (2.58\pm0.18)</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>(0.11\pm0.09)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>(1.27\pm0.12)</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>(0.18\pm0.03)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Included; \(^b\) not included; \(^c\) 21 IU/mL insulin; \(5 \mu\)mol/mL TD-34; \(25 \mu\)mol/L PAO; \(^d\) not detected.

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**Fig. 1. The Stability of TD-34 in HBSS at Different Time (\(n=3\))**
inhibited totally by the endocytosis inhibitor or the low temperature, we inferred that insulin transported across Caco-2 cell monolayers by multiple routes including passive diffusion.

When lowering the temperature from 37°C to 4°C, TD-34 was not detected, it indicated TD-34 transported across Caco-2 cell monolayers by a temperature dependent process. According to the inhibitor experiment, $P_{app}$ value of TD-34 decreased to 49% at least when the cells were treated with PAO, which further indicated transport of TD-34 across Caco-2 cell monolayers was mainly through an endocytosis process.

Basolateral side of Caco-2 cell monolayers was used to discuss the enhancement mechanism of TD-34 to avoid interference factors (transport cargoes and enzymes) distributed in apical side of Caco-2 cell monolayers. As TD-34 enhanced insulin delivery across Caco-2 cell monolayers for nearly 3 times in our previous reports, the effect of temperature on TD-34 enhancement activity was investigated. In this study, $P_{app}$ value (BL–AP) of insulin accompanied with 5 $\mu$mol/mL TD-34 was measured at 4°C and compared with previous results at 37°C. $P_{app}$ value (BL–AP) of insulin accompanied with TD-34 at 4°C decreased to 23% of that at 37°C (Fig. 2), indicating the temperature played a role in inhibiting the enhancement activity of TD-34 to some degree. Additionally, $P_{app}$ value (BL–AP) of insulin accompanied with TD-34 also increased 3 folds when compared to that of insulin alone at 4°C. This result demonstrated that the enhancement activity of TD-34 onto insulin delivery was temperature independent.

Since the low temperature restricted the fluidity of cell membrane, insulin could hardly transport across Caco-2 cell monolayers by a transcellular route. It indicated that TD-34 might enhance insulin delivery through a paracellular route.

**Effect of TD-34 on TEER of Caco-2 Cell Monolayers**

Paracellular permeability was limited by tight junction, and TEER values were used to represent the dynamic structure of tight junction in Caco-2 cell monolayers.

TEER value of Caco-2 cell monolayers was monitored during transport experiment for 24 h, and Caco-2 cells were exposed to transport medium for 2 h. TEER values of Caco-2 cell monolayers didn’t change significantly when Caco-2 cells were treated with 21 IU/mL insulin alone, but TEER value of Caco-2 cell monolayers decreased to 50% when Caco-2 cell monolayers were exposed to TD-34 accompanied with insulin for 2h (Fig. 3). Interestingly, TEER recovered to initial level gradually after TD-34 was removed (Fig. 3). The profile of TEER values was similar as our previous TEER experiment in which Caco-2 cells were treated with TD-34 alone. As reduction of TEER caused paracellular permeability increased, it testified TD-34 increased insulin diffusion across Caco-2 cell monolayers passively by loosening tight junction in a reversible way.

**Effect of TD-34 on the Tight Junction Protein**

Since loosened tight junction might cause TEER decreased in Caco-2 cell monolayers, we proposed that the tight junction of Caco-2 cell monolayers was loosened by TD-34 to enhance insulin paracellular delivery.

As this study was based on the previous reports that TD-34 enhanced the insulin delivery through the follicle route, the hair follicles are composed of stratified squamous epithelia, and the tight junction in stratified squamous epithelia might be modulated to enhance the hydrophilic molecules paracell-
lar delivery. As the epithelium tissue in deep follicle has the similar tight junction as that of Caco-2 cell monolayers, the changes in TEER of Caco-2 cells were reflected by the increased permeability of the paracellular pathway. Therefore, we speculated TD-34 might reduce the barrier function of the tight junction in follicle.

Claudins as tight junction proteins are the most important component of the barrier function and their expression levels demonstrated tissue-specific variations. Among the Claudins, immunofluorescence staining indicated Claudin-4 in epidermis of the skin is only expressed in the stratum granuloum, but Claudin-1 is expressed in the whole layer of epidermis in mouse and humans. Moreover, Claudin-1 as one of tight junction proteins was mainly distributed in all Caco-2 cell monolayers and maintained morphological integrity of Caco-2 cell monolayers. Thus, we believed Claudin-1 is important to prevent the insulin transdermal and transmembrane delivery. Based on the above considerations, we chose Claudin-1 and examine the effect of TD-34 on it. Distribution of Claudin-1 was analyzed in immunofluorescence experiments using confocal laser scanning microscope. Caco-2 cell monolayers were incubated in the following three groups. Group I, Caco-2 cell monolayers were incubated in DMEM for 2 h as control group (Fig. 4a). Group II, Caco-2 cell monolayers were treated with TD-34 for 2 h (Fig. 4b). Group III, Caco-2 cell monolayers were treated with TD-34 for 2 h, and then Caco-2 cells were continually incubated for another 22 h after TD-34 was removed (Fig. 4c). TD-34 significantly lowered the Claudin-1 immunoreactivity at the Caco-2 cell borders after Caco-2 cell monolayers were treated with TD-34 for 2 h. Interestingly, Claudin-1 immunoreactivity recovered after TD-34 was removed. Compared to TEER values, the reappearance of Claudin-1 was paralleled with the recovery of TEER. The reversible effect of TD-34 on both TEER and Claudin-1 localization suggested that TD-34 only transiently altered the tight junction structure of Caco-2 cell monolayers (Fig. 5). Furthermore, low cytotoxicity of TD-34 also ensured barrier functions recovery successfully and the correlation of TEER recovery with Claudin-1 reappearance provided an evidence for a role of TD-34 enhanced insulin across membranes by loosening the tight junction in a reversible way.

In summary, insulin alone transported across Caco-2 cell monolayers by multiple routes including passive diffusion. When insulin accompanied with a cationic cyclopeptide named TD-34, paracellular transport of insulin tended to become a main route across Caco-2 cell monolayers by loosening tight junction proteins in a reversible way. The sequence of TD-34, a cationic cyclopeptide, might be considered as a lead structure of cell penetrating peptides or percutaneous enhancers.

**Acknowledgments** This research was supported by National Natural Science Foundation of China (No. 30873448 and No. 21104008) and Fundamental Research Funds for the Central University (No. DUT13LAB07). The authors gratefully acknowledge the contribution of Dr. Fangling Ji in the revision of the manuscript.

**REFERENCES**


9) Kim YC, Ludovice PJ, Prausnitz MR. Transdermal delivery en-