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A novel approach using functional peptides for efficient intestinal absorption of insulin

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Abstract

The aim of this study was to evaluate whether oligoarginine, a cell-penetrating peptide (CPP), can improve intestinal absorption of insulin in rats. Peptides composed of six (R_6), eight (R_8) and 10 (R_{10}) residues of arginine were used as the CPP. No insulin absorption was observed following administration of insulin solution alone; however, insulin absorption increased dramatically after coadministration of the D-form of R_6 (D-R_6) and the L-form of R_6 (L-R_6) in a dose-dependent manner. The effects on insulin absorption were more pronounced for D-R_6 than for L-R_6. Among oligoarginines composed of six, eight, or 10 arginine residues, D-R_8 showed the strongest enhancing effects on insulin intestinal absorption. In contrast, intestinal absorption of other model hydrophilic macromolecules, interferon-β and fluorescein isothiocyanate-labeled dextran 4400, was not affected by coadministration with oligoarginine. Pretreatment by the effective dose of L-R_6 did not induce lactate dehydrogenase leakage or histological damage, suggesting that oligoarginine has no untoward effect on the intestinal mucosa. Our data demonstrate that coadministration of oligoarginine increases intestinal insulin absorption markedly without causing detectable damage in cellular integrity and that the covalent binding between insulin and oligoarginine is not necessary for this effect. We conclude that oligoarginines are likely to become powerful tools for overcoming the low permeability of insulin through the epithelial cell membrane, the major barrier to oral insulin delivery.

Keywords: Cell-penetrating peptide (CPP); Oligoarginine; Insulin; Intestinal absorption; Drug delivery

1. Introduction

During the past few decades, biotechnology has yielded biotherapeutic agents for the treatment of several diseases [1–3]. Biotherapeutic agents are mainly peptides and proteins and are often administered parenterally because of insufficient oral bioavailability caused by the low permeability through the intestinal mucosa associated with their hydrophilicity, high molecular weight, and susceptibility to enzymatic degradation [4–6]. Even insulin, one of the most widely prescribed peptide drugs, has a restricted delivery route by subcutaneous injections. However, patients find the oral forms of therapeutic drugs more attractive than the injectable forms because oral forms are more convenient and easier to use. To yield therapeutic activity, orally administered biotherapeutic agents must be absorbed efficiently from the intestinal lumen to the circulation without being metabolized extensively in the intestine. Several approaches are employed at present to improve mucosal permeation of insulin and other macromolecular drugs, including utilizing absorption enhancers [7,8], protease inhibitors [7,9,10], and carrier systems such as nanospheres [11,12], liposomes [13–15], emulsions [16,17], and hydrogels [18,19]. Although such approaches can be very successful in the laboratory [7–19], they have not yet been accepted widely by clinicians and regulatory bodies.

It is clear that strategies directed at overcoming the enzymatic barrier alone will have only limited success in the development of oral forms of peptide and protein drugs. High oral bioavailability is unlikely to be achieved unless one can increase the membrane permeability of macromolecules. Recent reports using peptides referred to as cell-penetrating peptides

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(CPPs) indicate that drugs that are poorly permeable through the cell membrane can be taken up efficiently by diverse cells without altering drug activity [20–22]. These CPPs include human immunodeficiency virus (HIV)-1 Tat-(48–60) [23]; penetratin derived from the third helix of the Antennapedia homeodomain protein [24]; and oligoarginine, an oligomer of arginine (one of the basic amino acids) containing six or more amino acids [25]. HIV-1 Tat and oligoarginine have an abundance of arginines in their amino acid sequences, and these peptides can be introduced into the intracellular space-mediated endocytotic pathway by interaction between the positively charged arginine and the negative charge caused by proteoglycan binding onto the cell membrane surface [26–30]. CPPs covalently bound to various molecules, such as protein [31], antibodies [32], and liposomes [27,33] are taken up efficiently by cells. CPPs can also translocate through the membranes of numerous cells because of their low cell specificity. Although there is only limited information about their effects on the membrane permeability of macromolecular drugs, one study reported that insulin transport across Caco-2 cells was dramatically increased by conjugation of insulin with Tat peptide [34]. We speculated that peptide and protein drug absorption from the intestine might be improved by using this behavior of CPPs. At present, the CPP strategy research lacks sufficient numbers of *in vivo* studies to demonstrate their therapeutic potential.

We evaluated whether CPPs can improve intestinal absorption of insulin in rats. The effect of CPPs on macromolecular intestinal absorption was further examined using interferon-β (IFN-β) and fluorescein isothiocyanate-labeled dextran 4400 (FD-4) macromolecules as model macromolecular solutes. Peptides composed of six (R_6), eight (R_8), and 10 (R_{10}) residues of arginine were used as the CPP. To assess the safety of CPP in the intestinal epithelium, we also measured the leakage of lactate dehydrogenase (LDH), an intracellular enzyme inherent in the enterocyte of the intestinal epithelium, and performed light microscopic observation of intestinal tissues after pretreatment with oligoarginine.

2. Materials and methods

2.1. Materials

Recombinant human insulin (26 IU/mg), 20% formalin neutral buffer solution, and LDH Test Wako were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-R_6 (RRRRRR, R: L-arginine), D-R_6 (rrrrrr, r: D-arginine), D-R_8 (rrrrrrrr), D-R_{10} (rrrrrrrrrr), FD-4, and sodium taurodeoxycholate were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). IFN-β (0.6×10^7 IU/vial) was kindly supplied by Toray Industries, Inc. (Kanagawa, Japan). All other chemicals were of analytical grade and commercially available.

2.2. Preparation of drug and oligoarginine solution

Specific amounts of recombinant human insulin were dissolved in 50 μL of 0.1 M HCl in polypropylene tubes. The insulin solution was diluted with 2.4 mL of phosphate-buffered saline (PBS, pH 7.4) containing 0.001% methylcellulose, which prevents the adsorption of insulin on the tube surface, and normalized with 50 μL of 0.1 M NaOH. The FD-4 solution was prepared at 2 mg/mL, and the IFN-β solution at 0.9×10^6 IU/mL, in PBS. Specific amounts of L-R_6, D-R_6, D-R_8, or D-R_{10} was measured in the polypropylene tubes, and an aliquot of drug solution was added to the tubes and mixed gently to yield a clear solution.

2.3. In situ absorption experiments

This research was performed at Hoshi University and complied with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals. Male Sprague–Dawley rats weighing 180–220 g were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). Animals were housed in rooms controlled between 23±1 °C and 55±5% relative humidity, and they had free access to water and food during acclimatization. Animals were fasted for 24 h before the experiments. Following anesthetization by intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), rats were restrained in a supine position on a thermostatically controlled board at 37 °C. Additional i.p. injections of sodium pentobarbital (12.5 mg/kg) were used every 1 h to maintain the anesthesia.

The ileum was exposed following a small midline incision made carefully in the abdomen, and its proximal-to-ileocecal junction segments (length=10 cm) were cannulated at both ends using polypropylene tubing. These were ligated securely to prevent fluid loss and returned carefully to their original location inside the peritoneal cavity. To wash the intestinal content, PBS warmed at 37 °C was circulated through the cannula at 5.0 mL/min for 8 min using an infusion pump (KD Scientific Inc., Holliston, MA, USA). The cannulation tubing was removed and the segments were then closed tightly; about 1 mL of perfusion solution remained in the segments. Rats were left on the board at 37 °C for a further 30 min to recover from the elevated blood glucose concentration resulting from the surgery described above. After the 30 min rest, 0.5 mL of drug-oligoarginine mixed solution or drug solution (control) was administered directly into the 6 cm ileal loop made from the 10 cm pretreated segment. The doses were 50 IU/kg body weight for insulin, 5 mg/kg for FD-4, and 2.25×10^6 IU/kg for IFN-β. For evaluating the effect of L- and D-R_6 on insulin intestinal absorption, the doses of oligoarginines were 6.25, 12.5, and 25.0 mg/kg body weight. For evaluating the effect of chain length of oligoarginine on insulin intestinal absorption, the doses of D-R_6, D-R_8 and D-R_{10} were 6.25, 8.3 and 10.35 mg/kg, respectively, and the molar concentration of these administration solutions was the same (2.6 mM).

The relative bioavailability of enterally administered insulin was calculated relative to the subcutaneous (s.c.) route. Briefly, an insulin solution was prepared by dissolving an appropriate amount of recombinant human insulin in PBS at an s.c. dose of 1.0 IU/kg body weight. To maintain the same physical conditions for the rats, the same surgery (ileal loop) was performed on

animals receiving s.c. insulin as on rats in the intestinal absorption study.

During the experiment, a 0.25 mL blood aliquot was taken from the jugular vein before and 5, 10, 15, 30, 60, 120, 180, and 240 min after dosing. Tuberculin syringes (1 mL) were preheparinized in the usual fashion by coating the syringe wall with aspirating heparin and then expelling all heparin by depressing the plunger to the needle hub. Plasma was separated by centrifugation at 13,000 rpm (13,400×*g*) for 1 min. Blood glucose concentration was measured with a glucose meter (Novo Assist Plus, Novo Nordisk Pharma Ltd., Tokyo, Japan) in the insulin studies and used to represent the biological activity of insulin. The biological activity of insulin is expressed as a percentage of the predose glucose concentration adjusted to the corresponding blood glucose concentration in the control group (insulin solution). The magnitude of the hypoglycemic response was calculated using the trapezoidal method as the area above the curve (AAC) for 0–4 h. The plasma insulin concentration was determined using an enzyme immunoassay (Insulin ICMA kit, Molecular Light Technology, Wales, UK). The total area under the insulin concentration curve (AUC) from 0–4 h was estimated from the sum of successive trapezoids between each data point.

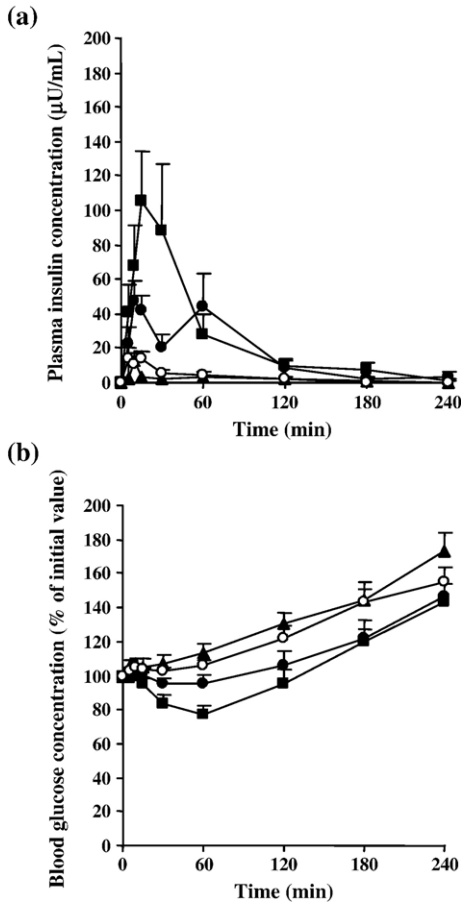

Fig. 1. Plasma insulin (a) and blood glucose (b) concentration vs. time profiles following in situ administration of insulin (50 IU/kg) with various dose of L-R_6 into the ileal segments. Each data point represents the mean±S.E. (n=3–8). Key: (○) insulin-PBS solution (control); (▲) 6.25 mg/kg; (●) 12.5 mg/kg; (■) 25.0 mg/kg of L-R_6.

The bioavailability was calculated relative to the s.c. injection as described above. The peak plasma concentration (C_{max}) and the time taken to reach the peak plasma concentration (T_{max}) were determined from the plasma insulin concentration–time curve. Plasma IFN-β concentration was determined using an enzyme immunoassay (IFN-β ELISA kit, Toray Industries, Inc., Kanagawa, Japan). Plasma FD-4 concentration was determined using a microplate luminometer (Mithras LB940, Berthold Japan, Tokyo, Japan) at excitation and emission wavelengths of 485 and 535 nm, respectively.

2.4. LDH leakage

The ileal loop from the pretreated segment after the in situ experiments, as described above, was used. The ileum was treated with 20 mL of PBS warmed to 37 °C and then flushed out with air. A solution of 0.5 mL PBS, 1% (w/v) sodium taurodeoxycholate, L-R_6 (25.0 mg/kg), or D-R_6 (25.0 mg/kg) was administered to the ileum and left in the ileal segments for 2 h. The ileal loop was washed with 3.0 mL PBS, and the intestinal fluid was collected. The concentration of LDH in the fluid was determined using the LDH Test Wako.

2.5. Light microscopy

The ileal segments were removed following the 4 h in situ absorption study and fixed with 20% formalin neutral buffer solution. Thin cross-sectional samples were prepared on a microtome and stained with hematoxylin and eosin for light microscopic observation to histologically assess tissue damage.

2.6. Statistical analysis

Each value is expressed as the mean±S.E. For group comparisons, analysis of variance (ANOVA) with a one-way layout was applied. The mean values were evaluated by Student's unpaired t test. $p<0.05$ was considered significant.

3. Results

3.1. Effect of oligoarginine on insulin absorption from the ileum

Figs. 1 and 2 show the effect of L-R_6 and D-R_6 on the ileal insulin absorption (A) and resultant hypoglycemic effect (B). No apparent hypoglycemic response was observed following administration of insulin solution, demonstrating no insulin absorption from the ileal segments. In contrast, coadministration of oligoarginine in a dose-dependent manner increased insulin absorption. As shown in Fig. 2, insulin absorption increased more after treatment with D-R_6 than after treatment with L-R_6, implying that the more metabolically stable oligoarginine induced the bigger insulin absorption from the ileum.

Table 1 summarizes the pharmacokinetic parameters derived from the insulin concentration–time profiles following in situ administration of insulin with oligoarginine to the ileal

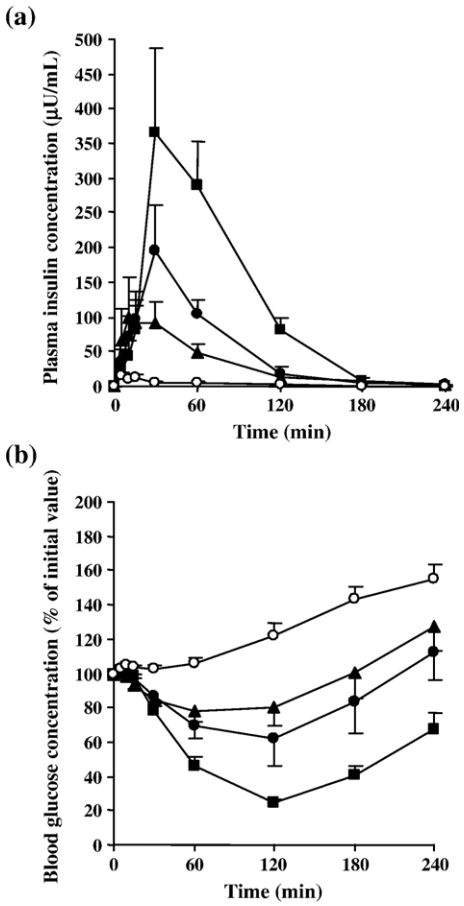

Fig. 2. Plasma insulin (a) and blood glucose (b) concentration vs. time profiles following in situ administration of insulin (50 IU/kg) with various doses of D-R_6 into the ileal segments. Each data point represents the mean±S.E. (n=3–8). Key: (○) insulin PBS solution (control); (▲) 6.25 mg/kg; (●) 12.5 mg/kg; (■) 25.0 mg/kg of D-R_6.

segments. AUC is derived from the plasma insulin concentration–time profile, and the area AAC is derived from the blood glucose concentration–time profile. The relative bioavailability (BA) and pharmacological availability (PA) were calculated from data obtained in the s.c. injection study. Although negligible absorption was observed with administration of the insulin solution, L-R_6 and D-R_6 significantly increased C_{max}, AAC, AUC, PA, and BA, which are all related to the extent of absorption. Significant linear relationships resulted after plotting AUC (r=0.721, p<0.01) and AAC (r=0.619, p<0.01) of the insulin vs. dose response to L-R_6. Similar patterns were observed for AUC (r=0.8328, p<0.01) and AAC (r=0.9074, p<0.01) of the insulin vs. dose response to D-R_6.

3.2. Effect of oligoarginine on IFN-β and FD-4 absorption from the ileum

Table 2 shows the effect of oligoarginine on the intestinal absorption of the protein drug, IFN-β. Although the same dose of L-R_6 (25.0 mg/kg) significantly increased the ileal absorption of insulin (Fig. 1, Table 1), the ileal absorption of IFN-β did not increase in the presence of L-R_6. Similarly, neither L-R_6 nor D-R_6 (25.0 mg/kg) increased the ileal absorption of FD-4, which is representative of hydrophilic macromolecules (Table 3).

3.3. Effect of chain length of oligoarginine on insulin absorption from the ileum

Insulin was coadministered with oligoarginine composed of six, eight, or 10 arginine residues (6.25, 8.3 or 10.35 mg/kg, respectively) to the ileum, and their effects on insulin absorption were compared (Fig. 3). As shown in Fig. 3, D-R_8 and D-R_{10} enhanced insulin absorption from the intestine more markedly than did D-R_6. Table 4 summarizes the pharmacokinetic parameters derived from the insulin concentration–time profiles shown in Fig. 3. D-R_8 caused the greatest increase in intestinal insulin absorption. Compared with D-R_6, D-R_8 had 3.4-times higher relative BA and D-R_{10} had 2.7-times higher relative BA for insulin.

3.4. Biochemical and histological examinations of the ileal membranes following oligoarginine administration

Table 5 shows data on LDH leakage in the absence (PBS, control) or presence of L- or D-R_6 (25.0 mg/kg) in the ileal loop for 2 h. LDH leaked negligibly into the mucosal side, and the

Table 1
Pharmacokinetic parameters following in situ administration of insulin with various doses of L- or D-R_6 into the ileal segments

	C_{max} (μU/mL)	T_{max} (min)	AAC (% glu. reduc. h)	AUC (μU h/mL)	PA (%)	BA (%)
Insulin solution	16.9±4.6	10.0±2.9	1.6±0.9	12.6±3.5	0.1±0.0	0.4±0.1
+ L-R_6						
6.25 mg/kg	24.0±12.6	10.0±2.9	0.2±0.2	10.1±2.2	0.0±0.0	0.3±0.1
12.5 mg/kg	64.1±9.1**	43.3±16.7	15.7±10.9	65.5±19.1	0.7±0.5	2.2±0.6
25.0 mg/kg	141.5±27.8*	25.0±8.2	37.9±12.0*	96.7±22.2*	1.7±0.5*	3.3±0.7*
+ D-R_6						
6.25 mg/kg	116.1±52.3	20.0±5.8	54.5±18.2	120.9±42.4	2.4±0.8	4.1±1.4
12.5 mg/kg	208.4±62.3	33.8±9.4	94.8±37.7	198.8±27.8**	4.2±1.7	6.7±0.9**
25.0 mg/kg	395.6±104.8*	37.5±7.5*	214.7±20.4**	464.0±109.1*	9.5±0.9**	15.7±3.7*

Data: mean±S.E. (n=3–8).
C_{max}, the maximum concentration; T_{max}, the time to reach the C_{max}; AAC, the area above the curve; AUC, the area under the curve; PA, pharmacological availability compared with s.c.; BA, relative bioavailability compared with s.c.
*p<0.05, **p<0.01, significant difference compared with the corresponding "insulin solution".

Table 2
Pharmacokinetic parameters following in situ administration of IFN-β with L-R_6 into the ileal segments

	C_{max} (IU/mL)	T_{max} (min)	AUC (IU h/mL)
IFN-β solution	10.0±4.1	103.8±53.8	39.2±17.1
IFN-β+L-R_6	6.4±1.2	50.0±10.0	11.9±3.8

Dose of L-R_6: 25.0 mg/kg.
Data: mean±S.E. (n=3–6).
C_{max}, the maximum concentration; T_{max}, the time to reach the C_{max}; AUC the area under the curve.

LDH leakage was similar in the oligoarginine-treated segments and the PBS-treated segments. In contrast, sodium taurodeoxycholate significantly increased LDH leakage (4.26 vs. 1.19 U).

Fig. 4 shows light micrographs of the ileal mucosal membranes pretreated with (a) PBS, (b) L-R_6 12.5 mg/kg, (c) L-R_6 25.0 mg/kg, and (d) L-R_6 37.5 mg/kg for 4 h. The micrographs (Fig. 4) show no apparent histological damage in the L-R_6-treated mucosal membranes compared with the PBS-treated (control) membranes. These data suggest that oligoarginine administration did not alter the ileal membrane integrity.

4. Discussion

In the past decade, studies have indicated that CPPs enable the delivery of different bioactive compounds and drug carriers by chemically hybridizing with target materials [27,31–33]. The CPPs deliver their cargo into the cytoplasm by directly perturbing the lipid bilayer structure of the cell membrane or by endocytosis [35,36]. CPPs appear to have little or no toxic effects because penetration causes little disturbance to membranes, and Tat appears to cause practically no harm to cell membranes [36,37]. Toxicity and undesirable side effects have not been detected in most in vivo applications of CPPs [31,36]. These methods are expected to become powerful tools for overcoming the low permeability of peptide and protein drugs through the epithelial cell membrane, which is the greatest barrier to oral delivery of macromolecular drugs. Although structural modification strategies using CPP may be useful, care must be taken not to reduce the biological activity of these drugs. So far, there is little information about whether intermolecular chemical conjugation is essential for a CPP's ability to induce cells to take up macromolecules.

Table 3
Pharmacokinetic parameters following in situ administration of FD-4 with L- or D-R_6 into the ileal segments

	C_{max} (μg/mL)	T_{max} (min)	AUC (μg h/mL)
FD-4 solution	0.3±0.1	105.0±90.0	0.8±0.9
FD-4+L-R_6	0.6±0.2	40.0±17.3	1.2±1.1
FD-4+D-R_6	0.7±0.2	120.0±60.0	2.0±1.1

Dose of L- or D-R_6: 25.0 mg/kg.
Data: mean±S.E. (n=3–4).
C_{max}, the maximum concentration; T_{max}, the time to reach the C_{max}; AUC, the area under the curve.

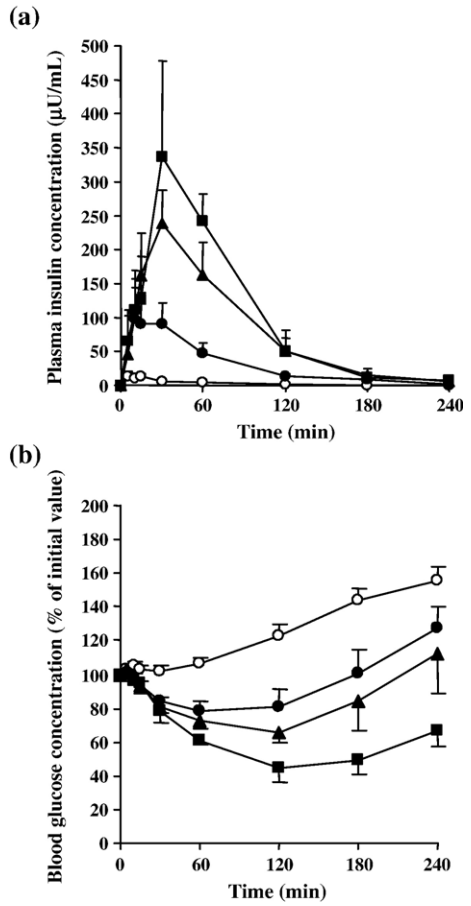

Fig. 3. Plasma insulin (a) and blood glucose (b) concentration vs. time profiles following in situ administration of insulin (50 IU/kg) with D-R_6 (6.25 mg/kg), D-R_8 (8.3 mg/kg) or D-R_{10} (10.35 mg/kg) into the ileal segments. Each data point represents the mean±S.E. (n=3–8). Key: (○) insulin PBS solution (control); (●)D-R_6; (■)D-R_8; (▲)D-R_{10}.

To address these issues, we examined whether oligoarginine enhances the absorption of intestinal bioactive macromolecular drugs without covalently binding to these molecules. As shown in Fig. 1 and Table 1, L-R_6 significantly enhanced intestinal insulin absorption and induced a strong hypoglycemic effect in a dose-dependent manner. More pronounced insulin absorption-enhancing effects were observed after D-R_6 coadministration with insulin (Fig. 2 and Table 1). To our knowledge, this is the first demonstration that insulin intestinal absorption can be enhanced by a physical mixture containing oligoarginines, and in particular, the D-form. Such mixture preparations are more feasible for delivering protein and peptide drugs than conjugated forms because they are unlikely to decrease the biological activity of the drug and because they are easy to prepare. These CPP strategies are considered highly promising for the development of an oral delivery system for insulin. In contrast, as shown in Table 2, L-R_6 had no enhancing effect on intestinal IFN-β absorption at a dose of 25.0 mg/kg, which was sufficient to increase intestinal absorption of insulin. These results suggest that the ability of oligoarginine to increase intestinal absorption might differ between peptide and protein drugs (molecular weight of insulin: ca. 5.8 kDa and IFN-β: ca. 23 kDa). Further study is needed using various macromolecules of different

Table 4
Pharmacokinetic parameters following in situ administration of insulin with D-R_6, D-R_8, and D-R_{10} into the ileal segments

	C_{max} (μU/mL)	T_{max} (min)	AAC (% glu. reduc. h)	AUC (μU h/mL)	PA (%)	BA (%)
Insulin solution	16.9±4.6	10.0±2.9	1.6±0.1	12.6±3.5	0.1±0.0	0.4±0.1
+ D-R_6	116.1±52.3	20.0±5.8	54.5±18.2	120.9±42.4	2.4±0.8	4.1±1.4
+ D-R_8	384.0±115.9	45.0±8.7*	222.8±43.8*	417.7±122.1*	9.9±1.9*	14.1±4.1*
+ D-R_{10}	238.4±49.3*	30.0±0.0	99.7±41.8	322.9±106.9	4.4±1.9	10.9±3.6

Doses of D-R_6, D-R_8, and D-R_{10}: 6.25, 8.3 and 10.35 mg/kg, respectively, and the molar concentration of these administration solutions was the same (2.6 mM).
Data: mean±S.E. (n=3–8).
C_{max}, the maximum concentration; T_{max}, the time to reach the C_{max}; AAC, the area above the curve; AUC, the area under the curve; PA, pharmacological availability compared with s.c.; BA, relative bioavailability compared with s.c.
*p<0.05, significant difference compared with the corresponding "insulin solution".

molecular size and charge to clarify the usefulness of CPPs as oral carriers for bioactive macromolecules.

The L- and D-oligomers of arginine have a similar ability to enter into cells [25]. However, we found that the ability of L- and D-oligomers of arginine to increase absorption differed dramatically in the small intestine. The reason for this difference could be explained by the observation that peptides containing the L-form of amino acids are metabolically more unstable than those containing the D-form [38]. Thus, L-R_6 may be degraded rapidly by peptidases that are highly active in the intestinal lumen.

The number of arginines within a peptide sequence has an important influence on the ability of that peptide to enter cells [22,25]. Futaki et al. [22] suggested that R_6 and R_8 are internalized best by mouse macrophage RAW264.7 cells of peptides composed of 4–16 residues of arginine. Incubation of Jurkat cells, a human T-cell line, with R15 resulted in more intensive internalization than other oligoarginines used [25]. Thus, one may speculate that the extension of an arginine oligomer does not by itself increase the degree of cellular uptake. We used in situ absorption experiments to evaluate the effect of the length of oligoarginine on insulin absorption. The efficiency of enhancement of bioavailability differed markedly between D-R_6, D-R_8, and D-R_{10}; D-R_8 enhanced insulin mucosal absorption the most efficiently. These results suggest that there is an optimum number of arginines for enhancing intestinal absorption of insulin (Fig. 3 and Table 4). Although the reason why there is an optimal number of arginine residues remains unknown, chain length is presumably associated with the degree of hydrogen bonding with phospholipids, interactions with the cell surface proteoglycans, or permeability of oligoarginine through the mucous layer. A more mechanistic study of the translocation of oligoarginine through cell membranes is required.

We found that the diffusive absorption of macromolecular dextran, FD-4, was not enhanced by coadministration with L- or D-R_6 at a concentration (25.0 mg/kg) sufficient to increase insulin absorption (Table 1). Because FD-4 is a hydrophilic compound and a marker of the paracellular pathway, these results exclude the possibility of involvement of opening the paracellular pathway on the insulin absorption-enhancing effect of oligoarginine. In addition, the microscopic observations together with the data on LDH leakage show that the membrane integrity appears to remain undamaged (Fig. 4 and Table 5). These data strongly suggest that the effect of oligoarginines on insulin absorption were not caused by cellular damage.

Table 5
Lactate dehydrogenase (LDH) leakage following PBS (control), L-R_6 (25.0 mg/kg), D-R_6 (25.0 mg/kg) or 1 w/v% sodium taurodeoxycholate pretreatment for 2 h

Preparation	LDH leakage (U)
Control	1.19±0.08
L-R_6	1.49±0.11
D-R_6	1.55±0.16
Sodium taurodeoxycholate	4.26±0.89*

Data: mean±S.E. (n=3–5).
*p<0.05 compared with control.

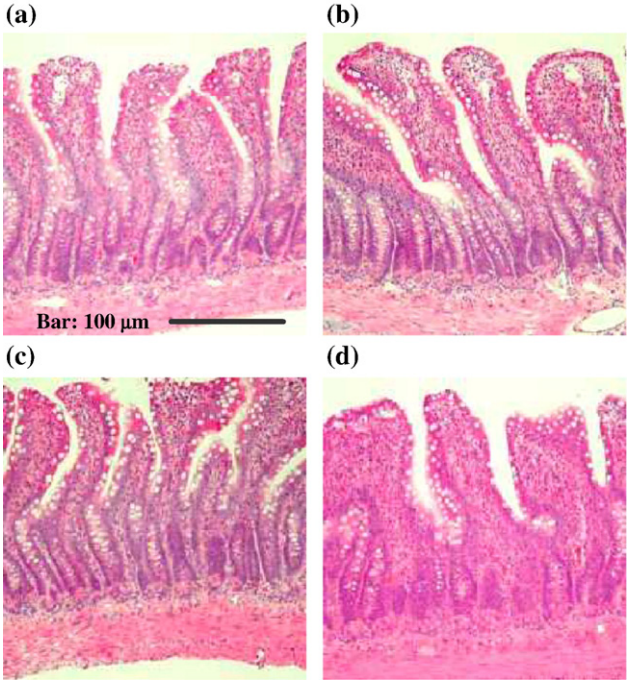

Fig. 4. Light micrographs of the ileal mucosal membranes following in situ administration of (a) insulin–PBS solution, and (b) 12.5 mg/kg, (c) 25.0 mg/kg, and (d) 37.5 mg/kg of L-R_6 with insulin for 4 h. The bar indicates 100 μm. Tissues were stained with hematoxylin and eosin following fixation in 20% formalin neutral buffer solution.

CPPs are thought to be taken up mainly by cell-mediated macropinocytosis [39] through the interaction between the positive charge derived from arginine residues and the negative charge derived from proteoglycans on the cell surface [26–30]. Therefore, we hypothesized that this proposed mechanism could explain how the molecular complex of the drug and oligoarginine is introduced into epithelial cells. We found that oligoarginine enhanced the intestinal absorption of insulin but did not enhance the absorption of other macromolecules such as IFN-β and FD-4. Insulin has a negative charge in neutral conditions, and insulin may associate with oligoarginine electrostatically. If so, these molecular complexes could be taken up by the interaction between oligoarginine and the surface of epithelial cells. In contrast, IFN-β has a relatively positive charge in neutral conditions, which may set up a repulsive force or prevent intermolecular interaction with oligoarginine. Similarly, noncharged macromolecules, such as FD-4, may not interact with positively charged oligoarginine. A more complete understanding of the mechanisms involved in the CPP-induced enhancement of insulin absorption through the intestinal membrane is needed.

5. Conclusions

We have demonstrated that intestinal insulin absorption is enhanced markedly without causing detectable damage in cellular integrity by coadministration of insulin with oligoarginine, but absorption of IFN-β and FD-4 was not enhanced by oligoarginine. The increase in absorption by oligoarginine occurred without chemical hybridization between oligoarginine and insulin. The detailed mechanism responsible for the ability of oligoarginine to increase insulin absorption remains unclear. To develop a macromolecular oral delivery system using oligoarginine, more data on its efficacy and safety and the underlying mechanism are needed.

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