Research Article

Penetratin-Mediated Transepithelial Insulin Permeation: Importance of Cationic Residues and pH for Complexation and Permeation

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ABSTRACT. Penetratin is a widely used carrier peptide showing promising potential for mucosal delivery of therapeutic proteins. In the present study, the importance of specific penetratin residues and pH was investigated with respect to complexation with insulin and subsequent transepithelial insulin permeation. Besides penetratin, three analogues were studied. The carrier peptide-insulin complexes were characterized in terms of size and morphology at pH 5, 6.5, and 7.4 by dynamic light scattering (DLS) and transmission electron microscopy (TEM), respectively. At pH 7.4 mainly very large complexes were present, while much smaller complexes dominated at pH 5. Presence of arginine residues in the carrier peptide proved to be a prerequisite for complexation with insulin as well as for enhanced transepithelial insulin permeation *in vitro*. Rearrangement of tryptophan residues resulted in significantly increased insulin permeation as compared to that of the parent penetratin. In general, pre-complexation with penetratin and its analogues at pH 5 gave rise to increased insulin permeation as compared to that observed at pH 7.4; this finding was further supported by a preliminary *in vivo* study using the parent penetratin.

KEY WORDS: Caco-2 cell culture model; carrier peptide; insulin; penetratin; transepithelial permeation.

INTRODUCTION

Peptide and protein therapeutics are highly potent and lead to predictive biological responses; thus, their use will likely provide better therapy for diseases that are not adequately treated by use of conventional small-molecule synthetic drugs, but at present, they require administration by injection, which is not optimal when taking patient compliance and costs into account. Oral delivery of peptide and protein therapeutics is, however, compromised by several factors including their chemical instability, large molecular size, and thus inability to cross biological barriers such as the intestinal epithelium.

Carrier peptides, some of which are known as cell-penetrating peptides (CPPs), are promising candidates as

enhancers of transepithelial permeation. Although they have primarily been explored for intracellular drug delivery of, *e.g.*, oligonucleotides (1) or antibodies (2), they may also be suitable candidates for transepithelial drug delivery involving non-covalent complexation with biopharmaceuticals (3). The primary sequence of a CPP is of great importance for its cell-penetrating efficiency. A high content of the cationic residues arginine (Arg) and lysine (Lys) facilitates initial electrostatic interactions with the negatively charged plasma membrane, which is important for subsequent membrane translocation (4,5). Furthermore, tryptophan (Trp) residues may also enhance interaction with the plasma membrane due to their affinity for cell-surface glycosaminoglycans (GAGs) (6). Penetratin, a 16-mer peptide with an amino acid sequence corresponding to the third helix of the antennapedia homeodomain protein (7), is a widely used and efficient CPP with a high content of Arg and Lys residues as well as two Trp residues. In an attempt to elucidate what makes the penetratin sequence unique in relation to its transmembrane delivery properties, Khafagi *et al.* investigated a range of penetratin analogues for their ability to enhance nasal insulin delivery in rats (8). This included the PenShuf analogue, in which only the positioning of cationic residues was conserved, and the analogues PenArg and PenLys, with Lys replaced by Arg or *vice versa*, respectively. The PenShuf analogue was shown to increase nasal insulin absorption significantly when compared to the parent penetratin.

Improved transepithelial delivery of peptide and protein therapeutics may be achieved *via* co-administration with a

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CPP (7–10) or by covalent conjugation to a CPP (11), of which the co-administration approach enables optimization of the drug-to-CPP mixing ratio thereby allowing for retained biological activity of the drug. However, the mechanism responsible for the capability of a CPP to mediate transepithelial delivery of a therapeutic peptide or protein, when co-administered as a simple mixture, is still not clear, but intermolecular interactions and the mixing ratio between CPP and drug have been proposed as important factors (9,10). Thus, in order to design future carrier peptides that interact with the plasma membrane in a way that leads to transepithelial delivery of a co-administered cargo, a deeper insight into the effect of formulation variables is needed.

In the present study, penetratin and its analogues (PenShuf, PenArg, and PenLys) were investigated to shed more light on the molecular factors influencing complexation between a carrier peptide and a therapeutic protein, using insulin as an example. Specifically, the experimental parameters that may affect this process were studied. The carrier peptides were mixed with insulin in different ratios and examined with respect to size distribution by dynamic light scattering (DLS) and morphology by transmission electron microscopy (TEM). Further, penetratin and its analogues were evaluated in order to identify sequence-specific features of penetratin that promote transepithelial permeation of insulin across Caco-2 cell monolayers when applied as complexes obtained at various pH and with varying carrier peptide-to-insulin ratios. Finally, an *in vivo* pilot study investigating the effect of formulation pH on the absorption of insulin from penetratin complexes was performed.

MATERIALS AND METHODS

Materials

Rink amide resin and coupling reagents for solid-phase peptide synthesis were purchased from Fluka (Buchs, Switzerland). All amino acid building blocks as well as other solvents and chemicals for peptide synthesis were purchased from Iris Biotech (Merkredwitz, Germany). Human recombinant insulin ($\geq 98\%$ purity) and other materials were obtained from Sigma-Aldrich (Buchs, Switzerland) unless stated otherwise.

Peptide Synthesis

Synthesis of penetratin and analogues thereof was carried out as previously described (12) by Fmoc solid-phase peptide synthesis (SPPS) using a MW-assisted

automated CEM Liberty synthesizer (CEM, Matthews, NC, USA). The peptides were purified by preparative RP-HPLC (250×21.2 mm Phenomenex Luna C18(2) column, 5 μ m). A linear gradient of eluent B (H₂O/MeCN 5:95, added 0.1% trifluoroacetic acid (TFA)) in eluent A (H₂O/MeCN 95:5, added 0.1% TFA) increasing from 0% to 45% over 25 min was applied at room temperature. The purity ($>95\%$) was confirmed by analytical RP-HPLC (150×4.6 mm Phenomenex Luna C18(2) column, 3 μ m) using a gradient from 0% to 60% of B over 30 min, applying UV detection at 220 nm. Molecular identity was confirmed by LC-HRMS using a Bruker MicroTOF-Q II Quadrapol MS detector. The peptides were lyophilized and stored at -18°C until further use. The synthesized penetratin and its analogues are listed in Table I.

Preparation of Insulin and Carrier Peptide-Insulin Complexes

In low-binding Eppendorf tubes, a minimum of 1 mg insulin was dissolved in 1 mL ultrapure water from a Barnstead™ water purification system (Thermo Scientific, Wilmington, NC, USA), and volumes of 50 μ L 0.1 M HCl were added to completely dissolve the insulin. The insulin solution was filtered through a 0.22 μ m Millex Millipore filter (EMB Millipore, Billerica, MA, USA) before the final protein concentration was determined by using a Nanodrop 2000c (Thermo Scientific, Wilmington, NC, USA). For the *in vitro* experiments, the insulin stock solution was further diluted to 10 μ M in Hanks balanced salt solution (HBSS) buffer (Gibco, Invitrogen, Naerum, Denmark) supplemented with either 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (mHBSS) (AppliChem, Darmstadt, Germany), and adjusted to pH 5 or 6.5, or 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (hHBSS) (AppliChem) and adjusted to pH 7.4; all media contained 0.05% (w/v) bovine serum albumin (BSA). A minimum of 1 mg of the carrier peptide was dissolved in ultrapure water in a glass vial before dilution with the respective HBSS buffers to 40, 60, or 80 μ M depending on the target carrier peptide/insulin ratio. Immediately before initiation of the experiment, the 10 μ M insulin stock solution was mixed 1:1 with a 40, 60, or 80 μ M carrier peptide stock solution ending up with a final carrier peptide/insulin molar ratio of 4:1, 6:1, or 8:1, respectively. For the *in vivo* experiments, insulin was administered at a dose of 1 μ mol/kg (167 IU/kg) alone or after complexation with 4 μ mol/kg penetratin in mHBSS or hHBSS and adjusted to pH 5 or 7.4, respectively.

Table I. Penetratin and Its Analogues

Name	Sequence	Mw (Da)	pI	Ref.
Penetratin	RQIKIWFQNRRMKWKK	2,246.7	12.31	(7)
PenShuf	R W F K I Q M Q I R R W K N K K	2,246.7	12.31	(8)
PenArg	RQIRIWFQNRR M R W R R	2,358.8	12.78	(8)
PenLys	KQIKIWFQN K K M KWKK	2,162.7	10.78	(8)

Altered residues in the analogues PenShuf, PenArg, and PenLys are presented in bold. (Molecular weight and isoelectric point are obtained from ExPASy.org.). MW molecular weight, pI isoelectric point

Optical Density

Optical density (OD) measurements of carrier peptide-insulin complexes were carried out on a POLARstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) in sealed black polystyrene 96-well plates (Thermo Fischer Scientific, Waltham, MA, USA) with a constant insulin concentration of 50 μ M. A 10-fold higher insulin concentration was employed for the OD measurements as compared to the *in vitro* experiments due to limitations in the sensitivity of the method. The measurements were performed on 100- μ L samples ($n=3$) containing complexes in ratios of 4:1, 6:1, and 8:1 each at pH 5, 6.5, and 7.4. OD readings were carried out every 10 min over 6 h at 405 nm at 37°C.

Dynamic Light Scattering

The approximate sizes of non-associated molecules and carrier peptide-insulin complexes were measured with a Zetasizer Nano ZS (Malvern Optics Instruments, Worcestershire, UK) employing a 633-nm laser. Size measurements were performed at various pH values ranging from 4 to 8 in a 10-mm cuvette with 173° angle detection. For each measurement, 10–20 runs of 10 s were performed. The insulin concentration was kept constant at 50 μ M, and measurements were performed on complexes in ratios 4:1, 6:1, and 8:1 for each of the pH values ($n=3$). A 10-fold higher insulin concentration was employed for the DLS measurements as compared to the *in vitro* experiments due to limitations in the sensitivity of the method. Size distributions were analyzed as distribution by volume.

Transmission Electron Microscopy

Penetratin-insulin complexes with a molar ratio 4:1, prepared at pH 5 and pH 7.4, were imaged with transmission electron microscopy as described previously (13). Briefly, 3–5 μ L of complexes formed with a concentration of 5 μ M insulin in suspension were loaded onto copper 400 mesh grids (Agar Scientific, Stansted, UK) coated with Formvar and carbon film. After 60 s, 10 μ L of distilled water was added, and subsequently excess water was removed. Thereafter, 10 μ L of 2% (w/v) uranyl acetate (Agar Scientific) was placed on the grid and left for 30 s. Finally, 20 μ L distilled water was added, and again excess water was removed. The grid was then left to dry. Images were acquired using a CM100 transmission electron microscope operating in an acceleration voltage range of 40–100 kV and at a magnification of approx. $\times 26,000$.

Cell Culture Model

Caco-2 cells were obtained from American Type Cell Cultures (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Naerum, Denmark) supplemented with 90 U/mL penicillin, 90 μ g/mL streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids, and 10% (v/v) fetal bovine serum (FBS) (Fischer Scientific, Slangerup, Denmark). Cells were grown in 5% CO₂ at 37°C and then detached from the culturing flasks at 80% confluency by trypsin-EDTA

treatment before being subcultured once a week. For permeability studies with well-differentiated cells in a tight monolayer, 1.0×10^5 cells were grown on polycarbonate membrane inserts (diameter 12 mm, area 1.13 cm², pore size 0.4 μ m, Corning Costar, Costar, NY, USA) in a 12-well Transwell® plate (Corning Costar) for 21 days.

In Vitro Transepithelial Insulin Permeation

Carrier peptide-insulin complexes and insulin formulations were prepared in the respective buffers at different peptide:insulin ratios as described above just before initiation of the experiment. As a control of the intact integrity of the Caco-2 monolayer during the experiments, the permeability of ³H-mannitol (1 μ Ci/mL in 10 mM hHBSS pH 7.4 added 0.05% (w/v) BSA) across the epithelium was evaluated.

Prior to the experiment, the culturing medium was removed by washing the cells with 2 $\times$ 1 mL receptor medium (10 mM hHBSS pH 7.4 added 0.05% (w/v) BSA) before the apical and basolateral compartments of the experimental setup were filled with receptor medium and left to equilibrate for 15 min at room temperature prior to measuring the transepithelial electrical resistance (TEER) as a control to ensure the integrity of the cell monolayer. The experiment was initiated after adding the respective sample formulations (500 μ L) to the apical side of the filter inserts, which were transferred to 12-well plates pre-filled with 1 mL of receptor medium at 37°C. In the experiments with ³H-mannitol, 100- μ L samples were withdrawn from the basolateral side of the epithelium at time points 0, 30, 60, 90, 120, and 180 min and immediately mixed with 2 mL Ultima Gold™ (Perkin Elmer, Waltham, MA, USA) before subjected to analysis using a scintillation counter (Packard Tri-Carb 2100 TR, Canberra, Dreieich, Germany). In the experiments where insulin measurements were performed, 100- μ L samples containing carrier peptide and/or insulin were withdrawn from the basolateral side of the epithelium at time points 0, 60, 120, and 180 min and kept on ice until quantification of insulin by employing an ELISA kit (Mercodia, Uppsala, Sweden) as described by the manufacturer using the absorbance measured at 450 nm on a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) for quantification. Withdrawn samples were immediately replaced with the same volume of receptor medium.

The apparent permeability coefficient (P_{app}) was calculated by using the equation:

$$P_{app}(\text{cm/s}) = dQ/dt \times 1/(A \times C_0) \quad (1)$$

where dQ/dt is the steady-state flux, A (1.13 cm²) is the area of the Caco-2 monolayer, and C_0 is the initial insulin concentration in the donor compartment.

Viability Assessment

The cellular viability of the well-differentiated Caco-2 cell epithelium was determined immediately after each transport experiment and assayed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)/phenazine methosulfate (PMS) assay

as previously described (14). Briefly, the epithelium was incubated with 0.32 mL of MTS/PMS solution (240 $\mu\text{g/mL}$ MTS, 2.4 $\mu\text{g/mL}$ PMS (Promega, Madison, WI, USA) in hHBSS) for 1.5 h with horizontal shaking protected from light at 37°C. The absorbance of samples (100 μL) from each filter insert was subsequently measured at 492 nm on a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany).

The relative viability was calculated by using the equation:

$$\text{Relative viability (\%)} = (A_{\text{sample}} - A_{\text{SDS}}) / (A_{\text{buffer}} - A_{\text{SDS}}) \times 100 \% \quad (2)$$

where A_{sample} is the absorbance of the samples, A_{SDS} is the absorbance of the positive control (0.2% (w/v) sodium dodecyl sulfate (SDS)), and A_{buffer} was absorbance of the negative control that corresponds to 100% cell viability.

Rat Intestinal Injection

Male Sprague-Dawley rats (Taconic, Ejby, Denmark) weighing 252 ± 7 g were allowed to acclimatize for at least 8 days at 20–22°C and 45–65% humidity with free access to water and food. The rats were fasted for 18 h prior to surgery. Hypnorm (fentanyl 0.315 mg/mL and fluanisone 10 mg/mL) (VetaPharma, Leeds, UK) and Dormicum (midazolam 5 mg/mL) (Nomeco, Copenhagen, Denmark) were mixed 1:1 (v/v) with ultrapure water and used for anesthesia. The subcutaneous (s.c.) initial dose was 2 mL/kg, and subsequent s.c. doses of 1 mL/kg were given every 20 min within the first hour and every 40 min thereafter to keep the animals anesthetized. The abdominal wall of the rats was cut open and the small intestine localized. PE-50 catheters (Smiths Medical, Keene, NH, USA) of a length of 10 cm were inserted into the middle of the intestine. The catheters were secured with 3/0 suture (Braun, Tuttlingen, Germany), and care was taken not to interrupt the mesenteric blood flow. The intestine was hydrated with 1–2 mL isotonic sodium chloride solution before the rat abdominal wall was closed by using the AutoClip Wound Closure System (Alzet, Cupertino, CA, USA) with 9-mm clips. The rats were left on a heating plate (Scandidact, Kvistgaard, Denmark) at 37°C for 30 min after surgery and prior to dosing of test formulations in order to recover from a potentially elevated blood glucose level resulting from stress induced by handling and surgery. Test solutions of 500 μL were injected directly into the mid-intestines of the rat through the catheter using a 1-mL syringe with a G25 needle inserted into the catheter. A volume of 200 μL air was injected immediately after dosing of the test formulations to fully empty the catheter. Blood samples (200 μL) were collected from the tail vein at time points 0, 5, 15, 30, 45, 60, 90, and 120 min after dosing. Samples were placed on ice in microcuvette vials (Sarstedt, Nümbrecht, Germany) containing EDTA in a final concentration of 10 mM, and upon cooling, they were centrifuged at $\times 2,000$ g for 10 min (Eppendorf centrifuge 5424, Eppendorf, Hamburg, Germany). Blood glucose was measured at time points of 15, 30, 45, 60, 90, and 120 min after dosing by using an On-Call blood glucose meter (Acon, San Diego, CA, USA). After the final blood sampling, the animals were euthanized while still

under anesthesia using 90% CO_2 in O_2 according to general recommendations (15). The abdominal wall of the rats was re-opened after the experiment, and it was checked that the catheters were still in place. Likewise, control of potential damage to the intestines was determined macroscopically. The experiments were conducted according to the National Animal Research Permit (reference number 2009/561-1622).

Data and Statistical Analysis

Data processing was performed in Microsoft Office Excel 2010 and GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed by using GraphPad Prism. Graphs and data in tables are depicted as mean $\pm$ standard error of mean (SEM) including variation between experiments, with n representing the total number of replicates. The statistical methods employed were t test and one-way analysis of variance (ANOVA). For *in vitro* permeability studies, viability studies, and TEER measurements, experiments were compiled from two passages, each with a total $n=3$ –6; for *in vivo* studies $n=6$ and for OD measurements and DLS $n=3$.

RESULTS

Formation Kinetics, Size, and Morphology of Carrier Peptide-Insulin Complexes

The sequences of penetratin and its three analogues studied are listed in Table I. The PenShuf analogue, displaying conserved positioning of cationic residues while other amino acids were shuffled, was included in the present study based on its earlier reported potential for enhancing transepithelial insulin delivery as compared to the parent penetratin (8). In order to elaborate on the influence of Arg *versus* Lys residues on complex formation and transepithelial permeation of co-administered insulin, the analogues PenArg (all Lys replaced with Arg) and PenLys (all Arg replaced with Lys) were included in the present study.

The level of complexation between carrier peptide and insulin in ratios 4:1, 6:1, and 8:1 at pH 5, 6.5, and 7.4 was investigated over 6 h by OD measurements (Fig. S1). Both increased pH and increased carrier peptide-insulin molar ratio influenced the degree of association, resulting in higher OD values. In all cases, an instant formation of complexes occurred with no changes in OD over time, except for the PenShuf analogue for which an increase in OD was observed over the experimental time period.

Complex Size Depends on Carrier Peptide, Ratio, and Formulation pH

To determine the influence of both pH and the carrier peptide-insulin molar ratio on the size of the resulting complexes, size distributions at pH 5, 6.5, and 7.4 for ratios 2:1, 4:1, 6:1, and 8:1 were analyzed by DLS (Fig. S2). In general, larger complexes were formed when the carrier peptide-insulin molar ratio was increased. In addition, for each carrier peptide-insulin combination, the complex size changed in a pH-dependent manner with the smallest complexes formed at pH 5 and the largest at pH 7.4. For

penetratin-insulin and PenShuf-insulin complexes, similar trends were observed, with the smallest complexes formed at pH 5 at a 2:1 ratio. A stepwise increase in complex size was seen as pH was raised to 6.5 and 7.4 as well as when the carrier peptide-insulin molar ratio was increased to 4:1 and 6:1. For PenArg-insulin complexes, an upper level in complex size was observed for a ratio of 8:1 at pH 6.5, whereas for PenLys-insulin, the upper size level was reached at a ratio of 6:1 at pH 7.4 as also found for penetratin-insulin and PenShuf-insulin. At pH 5, almost no complexes were formed between PenLys and insulin. Penetratin and PenShuf formed complexes with insulin having similar sizes at pH 5 and pH 7.4, whereas the penetratin-insulin complexes were approximately half the size of the PenShuf-insulin complexes at pH 6.5. The largest complexes were obtained with PenArg-insulin complexes at pH 6.5 and above, reaching sizes larger than 4,000 nm, while the PenLys-insulin complexes were the smallest with sizes just above 1,000 nm.

Since a clear tendency for pH-dependent complex formation between carrier peptide and insulin was evident, the level of small and large complexes in the pH range 5–8 was determined by DLS (Fig. 1). Data are shown for the 4:1 carrier peptide-insulin ratio, which has previously been described to increase the transport of insulin at pH 7.4 (9). The presence of small complexes was especially pronounced at low pH (Fig. S3), and their decline with increasing pH correlated with an increase in the volume of larger complexes.

Morphology of Penetratin-Insulin Complexes at pH 5 and 7.4

The penetratin-insulin complexes prepared in a ratio of 4:1 at pH 5 and pH 7.4 were imaged by TEM in order to obtain knowledge on the morphology of the complexes

formed. Representative images of the two samples are shown in Fig. 2, and it is evident from the images that the morphology of the few larger penetratin-insulin complexes formed at pH 5 appears to be denser than the complexes formed at pH 7.4, for which a looser network structure was observed. Also, higher numbers of smaller complexes were observed at pH 5 as compared to pH 7.4.

Penetratin and Its Analogues Mediate pH-Dependent Transepithelial Permeation of Insulin *In Vitro*

Penetratin, PenShuf, PenArg, and PenLys were evaluated for their ability to enhance transepithelial permeation of insulin across Caco-2 cell monolayers when using complexes formed at a penetratin-insulin 4:1 ratio (Fig. 3). As the surface pH of the jejunum is lower than that of the intestinal bulk lumen content (pH 7–8) (16), the permeation efficiency was evaluated at both pH 5, 6.5, and 7.4. Co-administration of insulin with penetratin at pH 7.4 or 6.5 did not result in increased insulin permeation, but decreasing the pH of the penetratin-insulin mixture to 5 resulted in a significant increase in the insulin permeation at all sampled time points (Fig. 3a). Also, when insulin was co-administered with PenShuf or PenArg, a significant pH-dependent increase in insulin permeation was observed when lowering the pH from 7.4 to 5 (Fig. 3b, c). With PenShuf, also a significant increase in the insulin permeation was observed when lowering the pH one unit to 6.5 (Fig. 3b). The presence of the PenLys analogue did not promote insulin transport at any of the three pH values tested (Fig. 3d). The PenShuf analogue was superior in increasing the transepithelial

Fig. 1. Mean sizes of small (black) and large (blue) carrier peptide/insulin complexes and the percentage distribution by volume after mixing in a 4:1 ratio (200 μ M carrier peptide, 50 μ M insulin) at varying pH. **a** Penetratin-insulin. **b** PenShuf-insulin. **c** PenArg-insulin. **d** PenLys-insulin. Results are shown as distribution by volume and mean $\pm$ SEM ($n=3$)

Fig. 2. TEM images of the penetratin-insulin formulations with a ratio of 4:1 at pH 5 (*left*) and pH 7.4 (*right*). Arrowheads: representative smaller complexes. Scale bars=1 μ m

permeation of insulin as compared to both penetratin, PenArg and PenLys; a trend clearly reflected in the P_{app} values listed in Table II. At a ratio of 4:1, PenShuf enhanced the insulin permeation 24-fold at pH 5, 10-fold at pH 6.5, and 3-fold at pH 7.4 as compared to the effect achieved with penetratin at pH 5. PenArg enhanced the insulin permeation to the same degree as penetratin at both pH 5 and pH 7.4, whereas PenLys lacked the ability to enhance insulin permeation as compared to administration of insulin without carrier peptide. Permeation of insulin as a result of administration alone was found to be virtually independent of the pH (Table II and Fig. S4).

Permeation Is Only Partly Dependent on the Carrier Peptide-Insulin Ratio at pH 5

When co-administering insulin with penetratin, PenShuf, or PenArg (Fig. 3a–c), a significant pH-dependent increase in insulin permeation was observed, with PenShuf yielding the highest permeation at pH 5. Hence, investigation of the effect of increasing the carrier peptide-insulin ratio from 4:1 to 6:1 was carried out at pH 5 (Table III and Fig. S5). However, this change in carrier peptide-insulin ratio from 4:1 to 6:1 did not significantly increase the amount of insulin that permeated the epithelium during co-administration with penetratin or

Fig. 3. Insulin transport across Caco-2 monolayers after incubation with 5 μ M insulin co-administered with 20 μ M **a** penetratin, **b** PenShuf, **c** PenArg, or **d** PenLys at pH 5 (*magenta*), pH 6.5 (*green*), or pH 7.4 (*black*). Data are presented as mean $\pm$ SEM ($n=3-6$). Levels of significance are *** $p<0.001$ and ** $p<0.01$ compared to pH 7.4

Table II. The Apparent Permeability Coefficient (P_{app}) for Insulin Permeation Across Caco-2 Monolayers After Administration of 5 μ M Insulin or the Same Amount of Insulin in Complexes with Penetratin, PenShuf, PenArg, or PenLys at pH 5, 6.5, or 7.4 in Ratio 4:1

	P_{app} (10^{-9} cm/s)		
	pH 5.0	pH 6.5	pH 7.4
Insulin	1.1 $\pm$ 0.0	n.a.	2.3 $\pm$ 0.3
Penetratin/insulin	6.9 $\pm$ 0.4	2.3 $\pm$ 1.1	2.5 $\pm$ 0.6
PenShuf/insulin	165.8 $\pm$ 0.4	71.6 $\pm$ 0.3	18.6 $\pm$ 0.1
PenArg/insulin	7.4 $\pm$ 1.3	3.4 $\pm$ 0.6	2.3 $\pm$ 0.5
PenLys/insulin	1.5 $\pm$ 0.8	2.5 $\pm$ 1.2	2.5 $\pm$ 0.8

Data are represented as mean $\pm$ SEM ($n=3-6$)

PenLys. By contrast, when employing PenShuf or PenArg, a carrier peptide-insulin ratio of 6:1 resulted in a slight but significant increase in insulin permeation as compared to that obtained for co-administration with carrier peptide in a 4:1 ratio. Thus, increasing the carrier peptide-insulin ratio from 4:1 to 6:1 resulted in an increase in insulin permeation by a factor of 1.7 for PenArg and 1.4 for PenShuf.

The PenShuf Analogue Decrease Cellular Viability and Epithelial Integrity at Low pH

To determine whether the pH-dependent increase in insulin permeation was associated with increased cellular toxicity, the cell viability was evaluated after each permeation experiment involving exposure to insulin-carrier peptide complexes. Furthermore, to evaluate the effect on the integrity of the cell layer, the TEER was determined before and after each experiment (Fig. 4b). No decrease in cellular viability was observed after incubation with penetratin, PenArg, or PenLys for any of the concentrations or pH values tested (Fig. S5). However, a significant decrease in cellular viability was observed at pH 5 after incubation with both 30 and 20 μ M PenShuf co-administered with 5 μ M insulin corresponding to ratios 6:1 and 4:1, respectively (Fig. 4a). This cytotoxicity elicited by PenShuf at pH 5 was concentration-dependent as a significantly lower viability was observed upon treatment with a PenShuf-insulin ratio of 6:1 as compared to a ratio of 4:1. Neither at pH 6.5 nor at pH 7.4 was a significant decrease in viability observed after incubation with PenShuf.

Table III. The Apparent Permeability Coefficient (P_{app}) for Insulin Permeation Across Caco-2 Monolayers After Administration of 5 μ M Insulin in Complexes with Penetratin, PenShuf, PenArg, or PenLys at pH 5 in Ratios 4:1 or 6:1

	P_{app} (10^{-9} cm/s)	
	Ratio 4:1	Ratio 6:1
Penetratin/insulin	6.9 $\pm$ 0.4	6.2 $\pm$ 1.4
PenShuf/insulin	165.8 $\pm$ 0.4	230.6 $\pm$ 9.5
PenArg/insulin	7.4 $\pm$ 1.3	12.4 $\pm$ 1.6
PenLys/insulin	1.5 $\pm$ 0.8	2.2 $\pm$ 1.0

Data are represented as mean $\pm$ SEM ($n=3-6$)

The results from the evaluation of the integrity of epithelial cell monolayers upon exposure to the complexes in the permeation experiments correlate well with the results of the viability assay, as no decrease in TEER was observed after incubation with insulin co-administered with penetratin, PenArg, or PenLys at all concentrations and pH values tested in the permeation studies (Fig. S6). On the other hand, a significant decrease in TEER was observed after incubation with both 20 and 30 μ M PenShuf co-administered with 5 μ M insulin at pH 5 (Fig. 4b). Hence, of the four carrier peptides employed in this study, only the PenShuf yielded insulin complexes exhibiting cytotoxic effects and that exclusively at pH 5.

Penetratin Mediates pH-Dependent Transepithelial Delivery of Insulin *In Vivo*

Finally, a preliminary *in vivo* study was performed to evaluate the ability of penetratin to enhance delivery of biologically active insulin across rat intestinal epithelium when using complexes formed at a penetratin-insulin 4:1 ratio (Fig. 5). Due to the toxic effect observed at pH 5 when using the otherwise superior PenShuf as carrier for insulin permeation, the PenShuf analogue was not included in this preliminary *in vivo* study conducted in order to validate the pH-dependent insulin permeation observed *in vitro*. The effect of administering the penetratin-insulin complexes prepared at pH 5 and pH 7.4 to the rat intestinal lumen correlated well with the observations *in vitro* (Fig. 3). A significant decrease in blood glucose to 50% of the initial level was observed 30–60 min after application of the penetratin-insulin complex prepared at pH 5 as compared to that prepared at pH 7.4, the latter not differing from insulin alone at pH 5.

DISCUSSION

Approaches to increase transepithelial delivery of insulin may involve use of carrier peptides, *e.g.*, CPPs (4,9,11,13–15), of which penetratin and its three analogues PenShuf, PenArg, and PenLys were investigated in the present study. Studies of the complex formation between these four potential carrier peptides and insulin showed that the smallest complexes were formed at the lowest tested carrier peptide-insulin ratio at pH 5, whereas the largest complex sizes were obtained with the highest examined carrier peptide-insulin ratio at pH 7.4. A different upper level in complex size was reached at different ratios depending on the specific carrier peptide (Fig. S2). Hence, both the carrier peptide-insulin molar ratio and the peptide sequence determine the size of the complexes formed, with Arg residues favoring formation of larger complexes. Exchanging all Arg residues with Lys residues resulted in significantly smaller complexes, indicating that the proportion of guanidinium groups, and not merely the net positive charge of the carrier peptide, constitutes the most important factor in complex formation with insulin. When measuring the size of the complexes formed with a carrier peptide-insulin ratio of 4:1 at different pH values, very small complexes were observed (Fig. 1). At pH 5, these small complexes dominated, with less than 1% of the larger complexes present, whereas at pH 7.4, mainly the larger

Fig. 4. **a** Cellular viability of Caco-2 cells after a 3-h permeation study with 5 μ M insulin co-administered with 20 μ M PenShuf at pH 7.4, 6.5, and 5.0 (ratio 4:1) or with 5 μ M insulin co-administered with 30 μ M PenShuf at pH 5.0 (ratio 6:1). **b** The relative transepithelial electrical resistance (TEER) measured after a 3-h permeation study under similar experimental conditions as for **a**. Results are shown as percent relative to control (HBSS pH 7.4) $\pm$ SEM ($n=6$). Levels of significance are *** $p<0.001$ compared to control; * $p<0.05$ and ** $p<0.01$ compared to PenShuf-insulin ratio 4:1 at pH 5.0

complexes were detected (Fig. S3). The exception was the PenLys-insulin formulation in which almost all molecules remained in small complexes at all pH values tested (Fig. 1d). The molecules in the penetratin-insulin formulation remained mainly in small complexes up to pH 6.5 (Fig. 1a), whereas the PenShuf-insulin and PenArg-insulin formulations contained no small complexes above pH 5.5. The pI of unfolded insulin is 5.3, whereas in the absence of a denaturing agent, the pI is 6.4 due to the masking of a carboxylate ionization as a result of interaction between the monomers (17). Hence, it is likely that the insulin pI under the experimental conditions employed during the DLS measurements approaches 6.4 rather than 5.3, and therefore, electrostatic interactions between the carrier peptides and insulin would be favored only at pH above 6.4. This would explain the abrupt shift in complex size around pH 6.5 for the penetratin-insulin and the PenArg-insulin complexes.

Fig. 5. Blood glucose level in rats following intraintestinal administration of 1 nmol/kg insulin at pH 5 (black) or co-administration of 1 μ mol/kg insulin (167 IU/kg) with 4 μ mol/kg penetratin at pH 5 (magenta) or 7.4 (green). Data are presented as mean $\pm$ SEM ($n=3-6$). The levels of significance are ** $p<0.01$, and * $p<0.05$ compared to insulin at pH 5

The TEM images revealed that at pH 5, only a few larger complexes were formed and that these appeared to be more dense than the complexes present at pH 7.4 (Fig. 2). This observation leads to the speculation that the observed complexes at pH 5 may consist of individual unfolded insulin molecules, as the pI of these approaches 5, and therefore, the solubility is decreased at pH 5. The overall lack of large complexes formed at pH 5 (Fig. 1) leaves a higher fraction of penetratin as free molecules in solution, which might contribute to the increased penetratin-mediated insulin permeation observed at pH 5 (Fig. 3a) as compared to that at pH 7.4 (Fig. 4). At pH 7.4, the penetratin and insulin molecules are oppositely charged, and therefore, electrostatic interactions contribute to the penetratin-insulin complexes formed, leaving less free penetratin molecules in solution for interaction with the epithelium. An alternative rationalization might be that insulin is not able to permeate the epithelium unless assisted by a carrier peptide, and then, it is more likely that smaller rather than larger carrier peptide-insulin complexes are able to facilitate diffusion across the epithelium.

Overall, the pI of the therapeutic peptide appears not to be the main determinant for complex formation with a carrier peptide at a given pH, since sequence-specific properties of the carrier peptide influences the size distribution, e.g., almost no larger complexes were present in the PenLys-insulin formulations at pH 6.5–7.4 (Fig. 1d) despite the opposite charges of insulin and PenLys. Moreover, only in the penetratin-insulin formulation, a shift from almost 100% small complexes to 100% large complexes was observed around pH 6.5 (Fig. 1c), albeit a shift toward larger complexes was also observed for the PenArg-insulin composition (Fig. 1c) at pH just above 5.5. Hence, in agreement with the findings by Kamei *et al.* (18), this study demonstrates that intermolecular interactions between carrier peptides and a therapeutic peptide or protein depend on the pI of the therapeutic peptide, indicating that electrostatic interactions play an important role in complex formation. Both Kamei *et al.* and Khafagi *et al.* have previously used

surface plasmon resonance (SPR) to study the importance of intermolecular interactions between a carrier peptide and a peptide drug when co-administered *in vivo*, showing that strong interactions facilitate transepithelial transport of the peptide drug (18–20). The present study adds to those studies by resolving which sequence-specific properties of the carrier peptide are determinants for the size of carrier peptide-insulin complexes prepared at different pH values. In addition, an inverse relationship between carrier peptide-insulin complex size and membrane permeability enhancement by the carrier was observed (Figs. 1 and 3).

Penetratin was evaluated for its ability to increase the transepithelial permeation of insulin *in vitro* via co-administration at pH 5, 6.5, and 7.4 revealing that penetratin-mediated insulin permeation was highly pH-dependent (Table II); a tendency further supported by a preliminary *in vivo* study in rat (Fig. 5). However, an effect, though unlikely, on the glucose level resulting from administration of penetratin alone was not examined in this preliminary *in vivo* study, but it will be investigated in future follow-up *in vivo* experiments. Examination of three penetratin analogues confirmed the tendency of pH dependence, as both co-administration with PenShuf and PenArg increased transepithelial insulin permeation *in vitro* (Fig. 3a, b) in the same pH-dependent manner as observed for the parent penetratin. However, the achieved improvement in PenShuf-mediated insulin permeation at pH 5 was found to be associated with a cytotoxic effect leading to a loss of epithelial integrity as reflected in a decreased TEER (Fig. 4b). Nevertheless, at pH 6.5 and 7.4, PenShuf significantly enhanced insulin permeation with no significant decrease in cell viability or loss of epithelial integrity (Fig. 4).

Among the carrier peptides employed in the present study, PenShuf was superior in enhancing the transepithelial insulin permeation *in vitro* with an up to 8-fold increase in the apparent permeability coefficient even at a formulation pH of 7.4 (Table II). A recent study underlines the importance of hydrophobic Trp residues for the cell-penetrating ability of a CPP (21), which was found to be augmented by an increased number of Trp residues that promote enhanced affinity in the interaction with cell-surface GAGs (6). Therefore, the promising results obtained in the present study with administration of PenShuf-insulin complexes *in vitro* may well be a result of the specific positioning of the Trp residues giving rise to an enhanced interaction with the surface of the Caco-2 epithelium leading to enhanced transepithelial insulin permeation. The toxic effect observed for PenShuf at pH 5 may therefore be an adverse effect of increased interaction between PenShuf and the cell membrane (Fig. 4a). On the other hand, no toxicity was observed at pH 6.5 and 7.4, which may be due to the fact that PenShuf and insulin form stable complexes at these pH values, thus protecting the Caco-2 cells from exposure to high local concentrations of non-associated PenShuf. Moreover, the PenShuf-mediated insulin permeation reached a plateau after 60 min at pH 6.5 and 7.4 (Fig. 3b). This is possibly a result of saturation in the otherwise highly effective PenShuf-mediated insulin permeation, whereas at pH 5,

disruption of the epithelial integrity occurred (Fig. 4) thereby giving rise to an apparent continuous facilitated insulin permeation across the monolayer (Fig. 3b).

Exchanging all Arg residues with Lys residues in the parent penetratin molecule had a highly negative effect on the insulin permeation *in vitro* (Fig. 3d). This was reflected in the P_{app} values obtained for insulin in the presence of PenLys, which were much lower than those determined in the presence of PenArg and penetratin (Table II). An increased number of arginine residues have previously been demonstrated to promote cellular uptake of CPPs (5,12), which most likely is a result of an improved hydrogen bonding between phospholipid headgroups in the cellular membrane and the CPP (7). However, earlier studies have also shown that an increased Arg content in a CPP negatively affects cell viability, possibly as a result of irreversible membrane perturbations mediated by the guanidinium groups (22,23). No decrease in viability was observed for PenArg in the concentrations employed in the present study (Fig. S6), and thus, the increased transepithelial insulin transport mediated by PenArg cannot merely be ascribed to a cytotoxic effect. Finally, increasing the molar ratio between the carrier peptides and insulin at pH 5 from 4:1 to 6:1 had a positive impact on the ability of PenArg to enhance insulin permeation. However, this trend was not seen for the parent penetratin or the PenLys analogue (Table III and Fig S5). Hence, (i) the pH, (ii) the specific positioning of the individual amino acid residues, (iii) the presence of guanidinium groups, and (iv) the molar carrier peptide-insulin ratio all appear to be contributing to the efficiency of selected highly cationic carrier peptides in facilitating transepithelial permeation of insulin.

CONCLUSIONS

In the present study, the carrier peptide penetratin and its analogues PenShuf, PenArg, and PenLys were investigated for their potential in enhancing the transepithelial insulin permeation after complexation under different experimental conditions. It was shown that the effect on insulin permeation is not merely dependent on physico-chemical characteristics of insulin and the carrier peptide, such as pI and amino acid composition, respectively, but also to a high degree on the resulting complex size, which proved to be pH-dependent. A high proportion of small complexes were present at the formulation pH that gave rise to the most efficient transepithelial insulin permeation.

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