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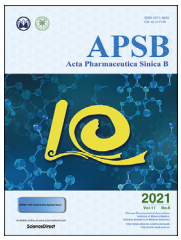

ORIGINAL ARTICLE

Effect of paracellular permeation enhancers on intestinal permeability of two peptide drugs, enalaprilat and hexarelin, in rats

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Abstract Transcellular permeation enhancers are known to increase the intestinal permeability of enalaprilat, a 349 Da peptide, but not hexarelin (887 Da). The primary aim of this paper was to investigate if paracellular permeability enhancers affected the intestinal permeation of the two peptides. This was investigated using the rat single-pass intestinal perfusion model with concomitant blood sampling. These luminal compositions included two paracellular permeation enhancers, chitosan (5 mg/mL) and ethylenediaminetetraacetate (EDTA, 1 and 5 mg/mL), as well as low luminal tonicity (100 mOsm) with or without lidocaine. Effects were evaluated by the change in lumen-to-blood permeability of hexarelin and enalaprilat, and the blood-to-lumen clearance of ^{51}chromium-labeled EDTA ($CL_{Cr\text{-}EDTA}$), a clinical marker for mucosal barrier integrity. The two paracellular permeation enhancers increased the mucosal permeability of both peptide drugs to a similar extent. The data in this study suggests that the potential for paracellular permeability enhancers to increase intestinal absorption of hydrophilic peptides with low molecular mass is greater than for those with transcellular mechanism-of-action. Further, the mucosal blood-to-lumen flux

Abbreviations: PE, permeation enhancer; $CL_{Cr\text{-}EDTA}$, clearance of ^{51}Cr-EDTA; P_{eff}, intestinal effective permeability; SDS, sodium dodecyl sulfate; SPIP, single-pass intestinal perfusion.

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of ^{51}Cr-EDTA was increased by the two paracellular permeation enhancers and by luminal hypotonicity. In contrast, luminal hypotonicity did not affect the lumen-to-blood transport of enalaprilat and hexarelin. This suggests that hypotonicity affects paracellular solute transport primarily in the mucosal crypt region, as this area is protected from luminal contents by a constant water flow from the crypts.

1. Introduction

Oral administration is preferred for systemically acting drugs because of its ease of intake and high patient compliance[1]. It also offers more flexibility in pharmaceutical formulation than other delivery routes and it comprises the bulk (62%) of total pharmaceutical products[2]. However, oral administration requires that the drug be chemically and metabolically stable in the highly dynamic gastrointestinal (GI) environment, and that it sufficiently permeates the intestinal membrane barrier. These features are typically associated with small molecules that are not substrates for various luminal digestive enzymes. Peptides, on the other hand, are larger and hydrophilic, resulting in low intestinal permeability. They are also substrates for luminal proteinases and peptidases, with very rapid luminal degradation rates[3–5]. Accordingly, albeit with a few exceptions, metabolic and/or permeation limitations hinder the development of oral pharmaceutical peptide products[6]. Nonetheless, there is a long and continuous interest in the oral administration route for peptides and other biologicals from the pharma industries, because these molecules are often superior to small molecules in their selectivity, potency, and safety[7]. For example, peptide drugs replace the physiological peptide hormones lacking in certain disorders, such as synthetic encephalin analogue in pain management and insulin in diabetes mellitus. However, many peptide drugs have a low permeation across cell membrane that prevents them from reaching and recognizing their intracellular targets[8].

Lately, pharmacokinetic and pharmacodynamic properties of some peptides have been improved by making them cyclic, so that their properties becomes similar to many low-molecular-mass drugs[9,10]. Pharmaceutical strategies for increasing intestinal peptide absorption have also been investigated[11]. For instance, chemical stability can be increased by enteric coating that prevents pH denaturation in the stomach, and metabolic degradation can be reduced by coadministration of peptidase inhibitors[12]. Colonic targeting has also been investigated, as the large intestine has lower peptidase activity[13]. Nevertheless, none of these biopharmaceutical approaches for increasing intestinal peptide stability has resulted in any clinical product, indicating that luminal stability is less of a formulation issue than the low intestinal permeability of these polar and larger peptides.

New chemical synthetic methods have improved the biopharmaceutical pharmacokinetic properties of peptides; they have also improved the target specificity by applying amino acid or backbone modifications and incorporation of non-natural amino acids. Conjugation of molecules has been used to reduce clearance and prolong terminal half-life, and/or improve physicochemical properties and aqueous solubility6. However, the molecular structure of these pharmaceutical peptides cannot readily be changed to increase intestinal membrane permeability without affecting their pharmacological effect. One approach to enhance permeation is to make the mucosal membrane transiently more permeable to the peptide. This may be achieved by incorporating permeation enhancers (PE), also called absorption-modifying excipients, into the drug formulation[14]. A PE can be transcellular and/or paracellular, depending on which transport route it affects[15]. Such a formulation strategy was recently approved for human use, in which the oral bioavailability of the peptide drug, semaglutide, was increased by formulating it with the transcellular PE, SNAC [sodium *N*-(8-(2-hydroxybenzoyl) amino)caprylate][16].

However, clinical use of PE has been largely unsuccessful. Semaglutide/SNAC is only one of two approved permeation-enhancing drug products, despite the number of preclinical reports investigating this formulation strategy. There is still no validated and robust formulation framework that can be used for rational pharmaceutical development of an oral dosage for any given peptide[12]. As there is an absence of approved oral peptide PE formulations this research was focused on improving the mechanistic understanding by using a rat single-pass intestinal perfusion (SPIP) method. This study monitored the effect of transcellular PE on the permeability of one smaller (enalaprilat, 348 Da) and one larger (hexarelin, 887 Da) peptide drug, *in vivo*[17]. Permeability of enalaprilat was substantially increased by sodium dodecyl sulfate (SDS) and caprate, while the jejunal permeability of hexarelin was unaffected. This is surprising as the relative PE-induced increase in drug permeability is reversely proportional to basal permeability of low-molecular-mass (350 Da) model drugs (enalaprilat>atenolol>metoprolol>ketoprofen) in both the rat SPIP and intraintestinal bolus models[18,19]. Possibly the increased membrane fluidity induced by the surfactant-like transcellular PEs is insufficient to compensate for the thermodynamic cost of partitioning into, and across, the epithelial apical cell membrane for a large, hydrophilic peptide like hexarelin. However, it remains to be investigated in the SPIP model if paracellular PEs affect the absorption of enalaprilat and hexarelin differently than PEs acting mainly on the transcellular route.

The objective of this study was to investigate the time-course effects of permeation enhancers on the peptides enalaprilat and hexarelin. Five luminal compositions, reported to increase paracellular permeability in the rat SPIP model, were tested[20,21]. These compositions included two paracellular PEs, chitosan (5 mg/mL) and ethylenediaminetetraacetate (EDTA, 1 and 5 mg/mL), as well as low luminal tonicity (100 mOsm) with or without lidocaine. Lidocaine was included because it potentiates the permeability enhancing effect of luminal hypotonicity[21]. Effects were evaluated in the rat small intestine by the change in lumen-to-blood permeability of hexarelin and enalaprilat, and blood-to-lumen clearance of ^{51}chromium-labeled EDTA ($CL_{Cr\text{-}EDTA}$), the clinical marker for mucosal barrier integrity.

2. Materials and methods

2.1. Active pharmaceutical ingredients and excipients, and other chemicals

The molecular structures and some physicochemical properties of enalaprilat and hexarelin are presented in Fig. 1 and Table 1[22,23]. Enalaprilat (Lot. No. 1235274), EDTA (Lot. No. E9884), lidocaine hydrochloride (Lot. No. PHR1257), hexarelin (Lot. No. 80666), Pefabloc SC (Lot. No. 76307), bovine albumin (Lot. No. 05470), and thiobutabarbital sodium salt hydrate (Inactin, Lot. No. T133) were purchased from Sigma−Aldrich (St. Louis, US). Sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$), potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH), and sodium chloride (NaCl) were purchased from Merck KGaA (Darmstadt, Germany). ^{51}Cr-EDTA was purchased from PerkinElmer Life Sciences (Boston, MA).

2.2. Study formulations

Seven different phosphate buffer perfusates were prepared—five at pH 7.4 (50 mmol/L), and two at pH 6.5 (8 mmol/L, Table 2). The five perfusates at pH 7.4 all contained 100 μmol/L enalaprilat and 90 μmol/L hexarelin. The two perfusates at pH 6.5 contained 200 μmol/L of enalaprilat and 180 μmol/L hexarelin instead. This was to enable plasma quantification of hexarelin in rat with our validated bioanalytical method, as the small intestinal permeability (and therefore plasma concentrations) of hexarelin reduces with pH[17].

Of the seven perfusates that all contained both enalaprilat and hexarelin, there were two isotonic (290 mOsm) control solutions that contained only buffer (1 at pH 6.5 and 1 at 7.4), and five test solutions (4 at pH 6.5 and 1 at pH 7.4) with compositions known to increase paracellular permeability[20,21]. Three of the five test solutions contained PEs: EDTA at 1 and 5 mg/mL, and chitosan at 5 mg/mL (these perfusate concentrations corresponds to oral doses of 0.2 and 1.0 g of a PE administered with 200 mL water)[18]. Two of the test solutions were hypotonic (100 mOsm), with or without lidocaine at 1 mg/mL; lidocaine is previously shown to potentiate the increase in paracellular permeability induced by hypotonicity[21]. All test solutions were at pH 7.4, except chitosan that was at pH 6.5 as it precipitates above this pH at luminally effective concentrations. All seven perfusion solutions contained the serine protease inhibitor, Pefabloc SC, at 0.3 mg/mL, as it completely inhibits the intestinal degradation of hexarelin in rat[23,24].

The perfusion solutions (100 mL) were prepared as described earlier[18]. There was no incompatibility, degradation, or binding to glass/plastic of any of the study drugs in solution (pH 6.5, 37 °C) during four 4 h (before:after, hexarelin: 29.9 *vs.* 33.9 μmol/L, enalaprilat 83.9 *vs.* 84.8 μmol/L). After addition of all perfusate constituents (*i.e.*, salt, PE, water) osmolarity was determined by freezing point depression using a Micro Osmometer (Model 3MO; Advanced Instruments, Needham Heights, USA).

2.3. Animals and study design

The surgical procedure and experimental setup of the rat SPIP experiments has been previously described in detail18. The study was approved by the local ethics committee for animal research (no: C64/16) in Uppsala, Sweden. In short, male Wistar Han rats (strain 273) from Charles River Co. (Germany), weight 299−395 g, were used. On the study day, the rats were anesthetized using an intraperitoneal injection of a 5% (*w*/*v*) inactin solution (180 mg/kg). Body temperature was maintained at 37.5 ± 0.5 °C. Systemic arterial blood pressure was continuously recorded by connecting the arterial catheter to a transducer operating a PowerLab system (AD Instruments, Hastings, UK) to validate the condition of the animal.

For the SPIP experiment, the abdomen was opened along the midline and a jejunal segment of 10−15 cm was cannulated, covered with polyethylene wrap, and placed on the outside of the abdomen. This avoids intestinal folding resulting in luminal pressure build-up, without affecting normal permeation parameters[25]. The bile duct was cannulated to avoid pancreaticobiliary secretion into the duodenum. After completion of surgery, ^{51}Cr-EDTA was administered intravenously as a bolus of 75 μCi (0.4 mL), followed by a continuous intravenous infusion at a rate of 50 μCi per hour (1 mL/h) for the duration of the experiment. During the first 30 min following surgery, each small intestinal segment was single-passed perfused with 37 °C phosphate buffered saline (6 mmol/L, pH 6.5 or 7.4). This allowed cardiovascular, respiratory, and intestinal functions to stabilize, and to achieve stable ^{51}Cr-EDTA activity in the blood plasma. The length of the proximal small intestinal segment was measured after the jejunal cannulation, and its wet tissue weight was measured after the experiment. The single-pass perfusion rate was at all times 0.2 mL/min (maintained by peristaltic pump, Gilson Minipuls 3, Le Bel, France).

Each perfusion experiment was divided into two parts. In the first part, the small intestinal segment was perfused with the isotonic control buffer solution (containing both peptide drugs at the same time, enalaprilat and hexarelin) during 60 min. In the second part of the experiment, the segment was perfused during 75 min with one each of the five test solutions (containing both peptide drugs at the same time, and one PE or

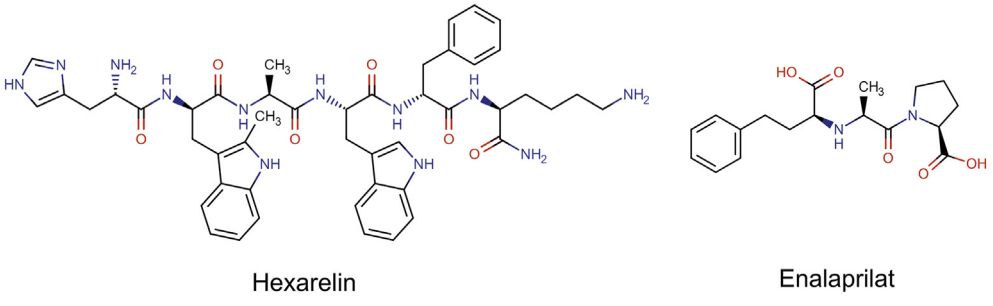

Figure 1 Molecular structures of two peptide drugs: hexarelin and enalaprilat.

Table 1 Physicochemical properties of the two study drugs: enalaprilat[22] and hexarelin[23].

Substance (BCS class)	MM (Da)	pK_a	PSA	HBA/HBD	Log$P$	Log$D_{7.4}$	Log$D_{6.5}$
Enalaprilat (III)	348	3.17[b]/7.84[a]	102.1	6/3	−0.13	−1.0	−1.0
Hexarelin (III)	887	−	300	9/11	0.73	−2.26	−3.40

HBA/HBD, hydrogen bond acceptor/donor; Log$D_{7.4/6.5}$, *n*-octanol−water partition coefficient at pH 7.4/6.5; Log$P$, *n*-octanol−water coefficient; MM, molar mass; p*K*a, dissociation constant; PSA, polar surface area.
[a]Acid.
[b]Base.

Table 2 Intestinal luminal composition of the seven perfusates investigated. The entering concentrations of enalaprilat, hexarelin, EDTA, and chitosan are presented, and so are the entering osmolarity and pH. There were two control solutions and five test solutions.

Perfusate composition	pH	Osmolarity (mOsm)	Enalaprilat/hexarelin concentration (μmol/L)
Control 1 pH 6.5	6.5	290	200/180
Control 2 pH 7.4	7.4	290	100/90
EDTA 1 mg/mL	7.4	290	100/90
EDTA 5 mg/mL	7.4	290	100/90
Chitosan 5 mg/mL	6.5	290	200/180
100 mOsm	7.4	100	100/90
100 mOsm + Lidocaine 1 mg/mL	7.4	100	100/90

100 mOsm±lidocaine). All five test solutions are previously shown to increase paracellular intestinal permeability in rat[18,20,21].

The five experiments were designed such that each rat served as its own control. The experimental period started with a rapid filling (<30 s) of the whole proximal segment of small intestine with the perfusate (about 1.5 mL for a 10-cm segment). The intestinal segment and perfusion solutions were kept at 37 °C and all perfusate leaving the segment was collected and weighed at each 15-min intervals. Previous investigations using this SPIP model without pharmaceutical excipients or PEs show that the integrity of the intestinal barrier is unaffected during at least 150 min, as there is no change in epithelial transport for a range of compounds: ^{51}Cr-EDTA, enalaprilat, atenolol, phenol red, acyclovir, and ketoprofen[26].

Blood samples of <0.3 mL (300 μL Li-heparin Sarstedt tubes) were collected from the femoral artery for a maximum volume of 4 mL during each experiment. Sampled blood volumes were replaced by an equivalent volume of saline (0.9% NaCl) solution with 70 mg/mL bovine serum albumin. Blood was sampled at 15-min intervals for 135 min (7 samples), put on ice, and centrifuged (5000×*g*, 3 min at 4 °C) within 10 min. 100 μL of the plasma was transferred to 0.5-mL micro tubes and stored at −80 °C until analysis. The bioanalytical ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC−MS/MS) method for quantifying enalaprilat and hexarelin in plasma is described elsewhere[17] and was used with slight modifications. The changes were: i) the analytical column was a Luna Omega Polar C18 (100 mm × 21 mm length × inner diameter, particle diameter 1.6 μm); and ii) the initial gradient was 2.0% B for 0.50 min, a linear increase to 90% B in 3.10 min, constant at 90% B for 0.80 min, then back to 2.0% B and constant again at 2.0% B for 1.50 min. The mobile phase constituents were: (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The concentration intervals in the calibration curve for the LC−MS/MS analytical method were 0.5−587 nmol/L and 1.5−113 nmol/L for enalaprilat and hexarelin, respectively. The accuracy was between 85% and 93% for hexarelin and between 101% and 105% for enalaprilat. The calibration curves were evaluated based on the back-calculated values, which were within ± 15% of their nominal values except at LLOQ where they were within ± 20% of the nominal value. The inter-day precision was in the range of 8.0%−9.7% for enalaprilat and 8.0%−15% for hexarelin.

2.4. *Determination of blood-to-lumen jejunal ^{51}Cr-EDTA clearance ($CL_{Cr\text{-}EDTA}$) and jejunal effective permeability (P_{eff})*

A detailed description of the calculation of blood-to-lumen $CL_{Cr\text{-}EDTA}$ and lumen-to-blood effective permeability (P_{eff}) can be found in Dahlgren et al. 2020[17]. In short, $CL_{Cr\text{-}EDTA}$ was calculated based on the transport of ^{51}Cr-EDTA from the blood to the lumen per 100 g jejunal tissue, while P_{eff} was calculated by relating the absorption rate from the lumen to the central circulation per jejunal surface are using the deconvolution method[27,28].

2.5. *Statistical analysis*

The sample size in each study group was six rats, on the basis of previous perfusion studies[18,29]. P_{eff} and $CL_{Cr\text{-}EDTA}$ values are expressed as mean ± standard deviation (SD) or standard error of the mean (SEM). The P_{eff} and $CL_{Cr\text{-}EDTA}$ ratio between the 45-min control and 60-min test periods in the rat perfusion studies are also presented (Eq. (1)).

$$\text{Ratio}\left(CL_{Cr-EDTA}\text{ or }P_{eff}\right)=\frac{\text{Mean value (test period)}}{\text{Mean value (control period)}} \quad (1)$$

The ratio was compared using the paired student's *t*-test with the Benjamini−Hochberg multiple *t*-test correction. Multiple comparisons between groups were performed using a one-way ANOVA with a post-hoc Tukey's multiple comparison test. Log transformation of values was performed when the original measured data were heteroscedastic and not normally distributed; this was investigated using the Bartlett test. Differences were considered to be statistically significant when the *P*-value was smaller than 0.05 ($P < 0.05$).

3. Results

3.1. *Plasma concentration−time profiles of enalaprilat and hexarelin*

The mean (±SEM) plasma concentration−time profiles of enalaprilat and hexarelin following the jejunal single-pass perfusions of the control solution (0−60 min) and five test solutions (60−135 min) are presented in Fig. 2a and b. These plasma data were used to determine baseline (control period) and test period P_{eff} values of enalaprilat and hexarelin, and their P_{eff} ratios (test *vs.* control period) using equation 1. There was a consistent increase in plasma concentration−time profile compared to the control period of both enalaprilat and hexarelin during the 75-min test period for EDTA and chitosan at 5 mg/mL. In contrast, there was no increase in the test period for EDTA at 1 mg/mL, or for the two perfusion solutions with hypotonicity (100 mOsm), with or without lidocaine (Fig. 2a and b).

3.2. *Blood-to-lumen jejunal ^{51}Cr-EDTA clearance profiles*

The mean (±SEM) $CL_{Cr\text{-}EDTA}$ over time following the jejunal single-pass perfusions of the control solution (0−60 min) and five test solutions (60−135 min) are presented in Fig. 2c. There was a consistent increase in $CL_{Cr\text{-}EDTA}$ during the 75-min test period for the two paracellular PEs: EDTA at 1 and 5 mg/mL, and chitosan at 5 mg/mL. Hypotonicity at 100 mOsm, both with and without lidocaine, increased $CL_{Cr\text{-}EDTA}$ sharply at the beginning of the 75-min test period. This was followed by a 50% recovery in $CL_{Cr\text{-}EDTA}$ during the last 30 min of the 75-min test period for 100 mOsm without lidocaine, and during the last 45 min for 100 mOsm with lidocaine (1 mmol/L, Fig. 2c).

3.3. *Lumen-to-blood effective jejunal permeability of enalaprilat and hexarelin*

The absolute mean (±SD) P_{eff} values of enalaprilat and hexarelin, with or without PEs, are presented in Table 3. The mean (±SEM) P_{eff} ratios of enalaprilat and hexarelin between the control and test period for the five test formulations are shown in Fig. 3a and b. The P_{eff} ratio (baseline *vs.* test) for enalaprilat and hexarelin increased significantly with EDTA and chitosan at 5 mg/mL. There was also a significantly higher P_{eff} ratio for enalaprilat and hexarelin when the EDTA concentration increased from 1 to 5 mg/mL. There was no increase in P_{eff} ratios for enalaprilat and hexarelin with EDTA at 1 mg/mL or with the two hypotonic (100 mOsm) perfusion solutions with or without lidocaine (Fig. 3a and b).

3.4. *Blood-to-lumen $CL_{Cr\text{-}EDTA}$*

The mean (±SEM) $CL_{Cr\text{-}EDTA}$ ratios (equation 1) between the control and test period for the five test formulations are shown in Fig. 3c. The $CL_{Cr\text{-}EDTA}$ ratio increased significantly for all test

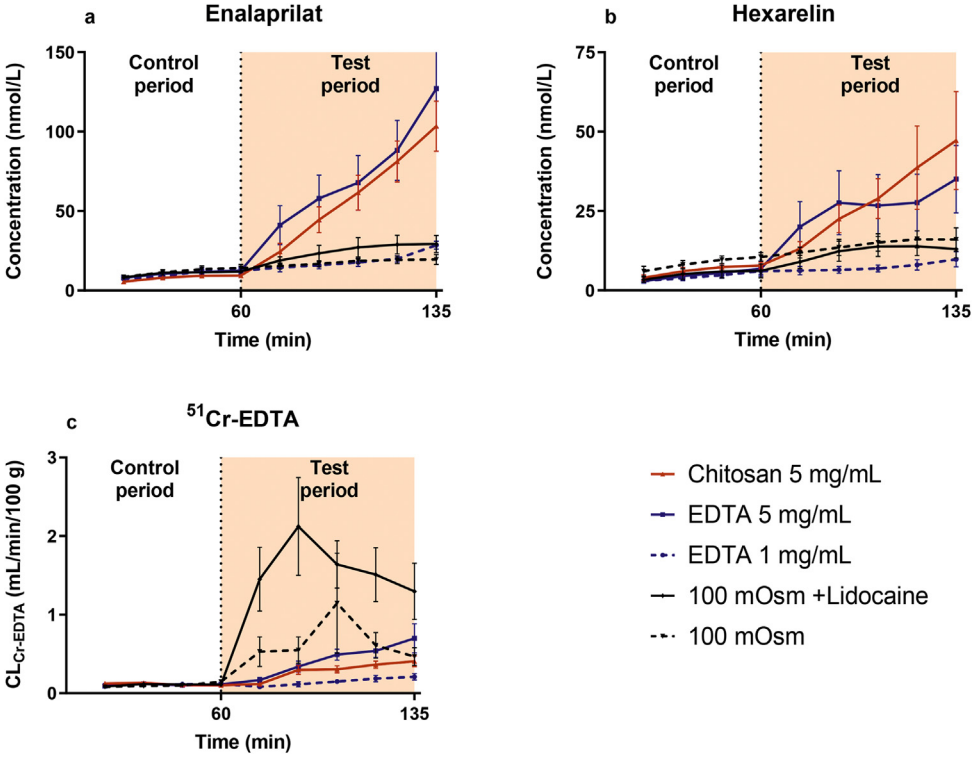

Figure 2 The mean ± SEM rat plasma concentration−time profiles ($n = 6$) of (a) enalaprilat and (b) hexarelin, and (c) the blood-to-lumen clearance of ^{51}Cr-EDTA ($CL_{Cr\text{-}EDTA}$), after intestinal perfusions of a control solution for 60 min, followed by a 75-min perfusion of any of five test solutions with constituents reported to increase paracellular permeability. There were three isotonic (290 mOsm) test solutions containing EDTA at 1 and 5 mg/mL, and chitosan at 5 mg/mL, and two hypotonic test solutions (100 mOsm) with or without lidocaine. All perfusates were at pH 7.4, except chitosan (pH 6.5). The control solution and all test formulations contained 100 µmol/L enalaprilat and 90 µmol/L hexarelin, except chitosan for which the concentration of both drugs were doubled (plasma values with the chitosan solution were normalized to the lower drug concentration).

solutions except EDTA at 1 mg/mL. The $CL_{Cr\text{-}EDTA}$ ratio also increased significantly by adding lidocaine at 1 mmol/L to the 100 mOsm luminal solution (*i.e.*, hypotonicity with lidocaine>hypotonicity).

3.5. *Transcellular versus paracellular intestinal permeation enhancers*

Fig. 4 compares the effect of paracellular and transcellular permeation enhancers on the small intestinal permeability of enalaprilat and hexarelin[17]. Both types of permeation enhancers increased the jejunal permeability of enalaprilat to similar extents. In contrast, only the small intestinal permeability of hexarelin was increased by the permeation enhancers mainly acting on the paracellular transport route.

4. Discussion

Intestinal singe-pass perfusion systems are commonly applied for various types of drug transport and absorption investigations[10,30,31]. Our laboratory has extensively investigated the *in vivo* effect of permeation enhancers (PE) on transport of model drugs and marker compounds with molecular masses ranging from 250 to 900 Da in the small and large intestine[14,17–20,26,29,32]. In this sequence of papers, the effect of a range of transcellular and paracellular PEs were initially examined with both the established rat single-pass intestinal perfusion (SPIP) model[18], and with the *in vivo* relevant rat and dog intraintestinal bolus absorption models[19]. A follow-up rat SPIP study investigating time dependence in PE effects showed that the lower effect in the rat bolus model compared to the SPIP model was attributed to time to maximum effect of the PE (not maximum effect)[26]. More specifically, the onset time of the PE on the intestinal mucosa needed to be shorter than the intestinal transit rate, which is identified as a crucial *in vivo* finding. We have also shown that physiological parameters, such as enteric neural activity, can have a substantial impact on results obtained from PE investigations using the rat SPIP model[29]. Furthermore, regional intestinal investigations showed that PE effects on drug permeability were similar in the small and large intestine, while mucosal injury was greater in the large intestine. This indicates permeation enhancing oral or rectal drug delivery systems targeting the large intestine might be less appropriate[20]. Finally, we have shown that the effect of surfactant-based PEs (caprate and SDS) was related to the free dissolved fraction (non-particulate or non-solubilized in micelles) in the intestinal lumen and not to the total dose[17,32], and that the same transcellular PEs had no effect on the transport of larger peptides[17]. All these experimental studies clearly demonstrate the importance of considering *in vivo* system with neural and endocrine functions and feed-back systems. The present study is a continuation of these *in vivo* investigations of PEs, where the main objective was to investigate the effect of paracellular enhancement of the transport of peptides of different sizes.

Pharmaceutical peptides are typically rapidly degraded in the gastrointestinal (GI) lumen and have low and highly variable intestinal permeability. This results in a low and variable rate and extent of intestinal absorption[33]. As a consequence of these drug delivery hurdles, there are only two approved, orally-administered, and systemically-acting peptide drugs, desmopressin and semaglutide. They are approved as they have very useful pharmacodynamic properties, despite the low bioavailability of the given oral dose (desmopressin, 0.1%; semaglutide, 0.4%−1.0% with SNAC) and high interindividual variability[34,35]. Semaglutide, a modified GLP-1 agonist (4113 Da) has a very long terminal half-life in plasma (∼1 week) and is significantly more potent than the endogenous GLP-1[36,37]. In addition, oral semaglutide is included in a drug product containing excipients that increase its luminal stability as well as its intestinal permeability. It is also the first product on the market using a PE to increase oral drug absorption. The manufacturer claims that the mechanism for permeation enhancement is the insertion of the PE (SNAC) in the intestinal epithelium, thereby fluidizing the lipid cell membrane to increase transcellular drug transport[16]. The approval of oral semaglutide has spurred interest in developing other oral formulations that increase the safe transport of peptides across the GI epithelium[38].

Transcellular PEs also tend to be more potent than paracellular ones, as seen for the model compounds atenolol, enalaprilat, FD-4/10, and ^{51}Cr-EDTA[14,39]. The rat SPIP model illustrates this well: SDS and caprate increase intestinal permeability more than permeation enhancers primarily reported to increase paracellular transport, like chitosan and EDTA[18,20]. Drug transport through the paracellular route is restricted because of the very low surface area available and the complex sequence of transport barriers that need to be crossed, such as tight junctions (zonula occludens), adherens junctions (zonula adherens) and desmosomes (macula adherens)[1,40]. We have also observed that the increase in small intestinal permeability is reversely proportional to the basal permeability of a range of low molecular mass (230−350 Da) model drugs (enalaprilat>atenolol>metoprolol>ketoprofen) in both the rat SPIP and intraintestinal bolus models[18,19]. This relationship, and the greater potency of transcellular PEs, support their use for increasing intestinal absorption of bulky and hydrophilic peptides.

As the intestinal permeability of peptides is very low, a higher proportional PE-induced increase in their permeability is expected compared to similar molecules with high basal permeability. Therefore, it is surprising that a recent rat SPIP study shows a substantial increase in the permeability of enalaprilat induced by transcellular PEs, but no effect on hexarelin. This is unexpected given that the basal permeability of hexarelin is > 10-fold lower than that of enalaprilat (0.0019 ± 0.0013 *vs.*

Table 3 The absolute effective permeability of enalaprilat and hexarelin in the control period (buffer at pH 7.4 and 6.5), and the five different test periods: three isotonic (290 mOsm) test solutions containing EDTA at 1 and 5 mg/mL, and chitosan at 5 mg/mL, and two hypotonic test solutions (100 mOsm) with or without lidocaine (L).

Perfusate composition	Effective permeability (× 10^{-4} cm/s)[a]	
	Enalaprilat	Hexarelin
Control 1 pH 6.5	0.009 ± 0.003	0.018 ± 0.0.6
Control 2 pH 7.4	0.010 ± 0.005	0.016 ± 0.01
EDTA 1 mg/mL	0.014 ± 0.003	0.017 ± 0.008
EDTA 5 mg/mL	0.069 ± 0.036	0.068 ± 0.055
Chitosan 5 mg/mL	0.057 ± 0.024	0.082 ± 0.064
100 mOsm	0.010 ± 0.005	0.032 ± 0.017
100 mOsm+L	0.017 ± 0.009	0.029 ± 0.015

[a]Data are mean ± SD. All test perfusates were at pH 7.4, except chitosan (pH 6.5). $n = 6$ for all groups except Control at pH 7.4 where $n = 23$.

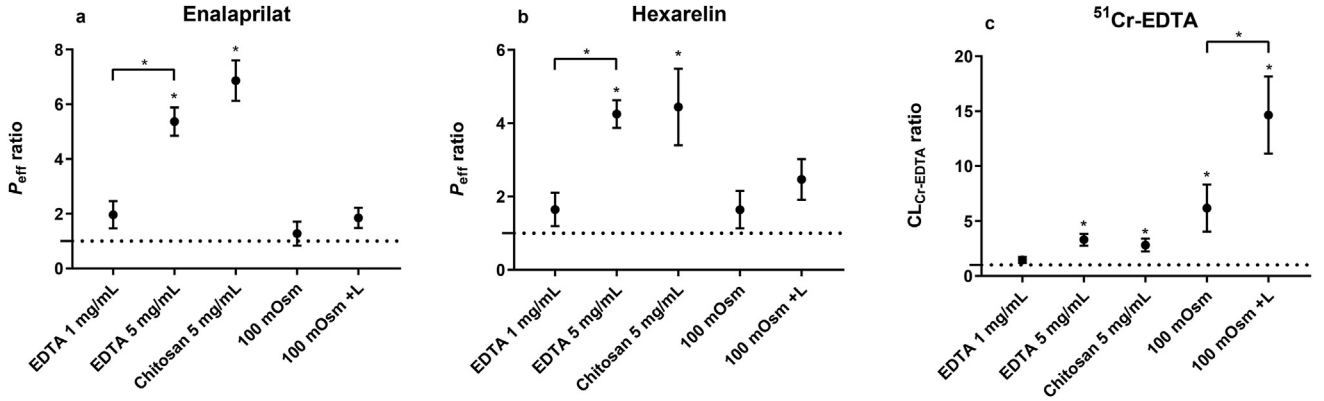

Figure 3 The mean ± SEM lumen-to-blood intestinal effective permeability (P_{eff}) ratio of (a) enalaprilat and (b) hexarelin, and (c) the blood-to-lumen ^{51}Cr-EDTA clearance ($CL_{Cr\text{-}EDTA}$) ratio, after the intestinal perfusions of a control solution for 60 min, followed by a 75-min perfusion of any of five test solutions with constituents reported to increase paracellular permeability. There were three isotonic (290 mOsm) test solutions containing EDTA at 1 and 5 mg/mL, and chitosan at 5 mg/mL, and two hypotonic test solutions (100 mOsm) with or without lidocaine (L). All perfusates were at pH 7.4, except chitosan (pH 6.5). A * represents a significant increase in P_{eff} or $CL_{Cr\text{-}EDTA}$ ratio.

$0.013 \pm 0.009 \times 10^{-4}$ cm/s)[17]. We speculated that the membrane fluidizing effect of SDS and caprate is insufficient to accommodate any transcellular transport of the larger peptide, hexarelin. In other words, the thermodynamic cost of partitioning into, and across, the SDS and caprate fluidized epithelial cell membrane is still too large for hexarelin compared to enalaprilat[41]. If this assumption is true, it may partly explain why PE strategies for increasing oral peptide delivery and absorption have largely failed to translate from preclinical evaluation to clinical product. However, it is possible that paracellular permeation enhancers would still be effective at increasing the permeability of hexarelin. This is because paracellular PEs primarily take into consideration the hydrodynamic diameter of the molecule in relation to the size of the paracellular pores, as opposed to lipoidal translocation with transcellular PEs. We tested this hypothesis in this paper to better understand how to improve GI peptide absorption[38].

Indeed, both paracellular PEs—EDTA at 5 mg/mL and chitosan at 1 and 5 mg/mL—increased jejunal permeability of hexarelin over time. This change was irrespective of their different mechanisms of action. The cations in chitosan interact with the epithelial membrane causing structural reorganization of tight junction proteins, whereas EDTA binds extracellular Ca^{2+} thereby limiting the impact of this ion on tight junction regulation[39,42]. It is interesting to note that despite the generally lower effect of paracellular permeation enhancers compared to transcellular ones, only the former had any impact on the epithelial transport of hexarelin[14,39]. Paracellular enhancers may consequently improve oral peptide delivery more than transcellular ones. However, it cannot be excluded that this effect is limited to hexarelin. Therefore, model peptides varying in molecular mass, hydrodynamic size, and other physicochemical properties must be investigated to further develop oral strategies to deliver peptides. It may also be that each oral peptide delivery system with a PE needs to be carefully designed and titrated. For instance, semaglutide with a SNAC dose of 300 mg was effective in humans, while a higher SNAC dose of 600 mg had no effect[16].

Perfusion of the intestinal segment with a hypotonic solution (100 mOsm) increased $CL_{Cr\text{-}EDTA}$ 6-fold in this study. This increase in paracellular permeability is likely due to a physiological feedback mechanism that moderates paracellular interstitium-to-lumen flux of osmolytes in response to luminal hypotonicity[43–45]. Luminal hypotonicity induces release of serotonin

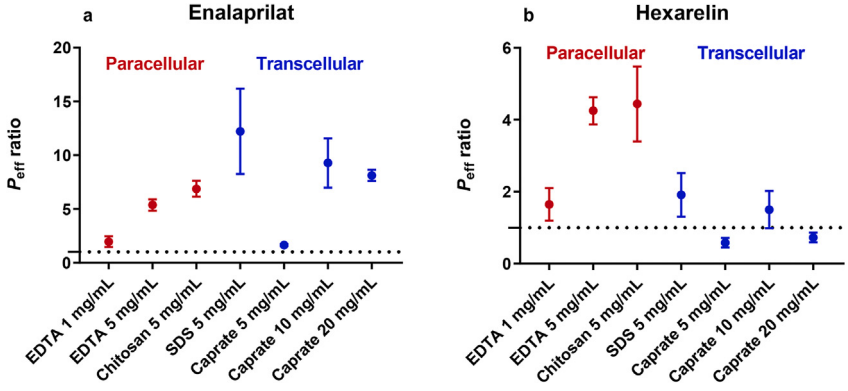

Figure 4 Comparison of the effect of paracellular (red) and transcellular (blue) permeation enhancers on the lumen-to-blood intestinal effective permeability (P_{eff}) ratio of (a) enalaprilat and (b) hexarelin, after the intestinal perfusions of a control solution for 60 min, followed by a 75-min perfusion of test solutions with permeation enhancers. The paracellular permeation enhancer data for EDTA and chitosan were from this study. The transcellular permeation enhancer data was recently published by us[17].

by the enterochromaffin cells in the intestinal mucosa and activates cholinergic efferent neurons involved in regulating jejunal mucosal permeability and secretion. Support for this view is the previous finding that luminal serotonin receptor antagonists, and nicotinic acetylcholine receptor antagonists, significantly reduce or abolish the hypotonicity-induced increase in duodenal mucosal paracellular permeability[45–47].

The hypotonicity-induced increase in mucosal permeability and osmolarity-adjusting capability may be enhanced by lidocaine, an amide-type of local anaesthetic. Adding lidocaine to the luminal hypotonic perfusate increased the permeability from six-fold to 15-fold in the present study, in line with similar increases shown previously[21]. The mechanism for the potentiating effect of lidocaine is presently not known. However, it is most likely not associated with blockade of voltage-gated Na^+ channels, as a selective blocker of this channel (tetrodoxin) inhibits the effect of hypotonicity[46]. It is also known that the local anaesthetic effects of lidocaine are rapid and of medium duration when administered either as a parenteral or topical formulation[48]. Luminal hypotonicity, with or without lidocaine, resulted in a sharp increase in $CL_{Cr\text{-}EDTA}$ followed by a recovery of about 50% during the intestinal hypotonicity exposure. Further, unlike the two paracellular permeation enhancers, the increase in $CL_{Cr\text{-}EDTA}$ induced by hypotonicity, with or without lidocaine, did not increase permeability of enalaprilat and hexarelin. This is in stark contrast to previous rat SPIP studies investigating paracellular and transcellular PEs. In those studies, there was a linear correlation between an increase in $CL_{Cr\text{-}EDTA}$ and model drug permeability[18,29]. A possible explanation for this is that luminal hypotonicity affects the physiological regulation of paracellular permeability primarily in the mucosal crypts; these crypts are largely unavailable to luminal contents and drugs, as there is a constant outward water flow to protect stem cells in this mucosal region[49]. This would explain why hypotonicity increase blood-to-lumen $CL_{Cr\text{-}EDTA}$—but not lumen-to-blood drug permeability—-whereas chitosan and EDTA affect paracellular permeability in the whole epithelium, and consequently transport of solutes in both directions.

5. Conclusions

In conclusion, this rat SPIP study showed that two permeation enhancers with mainly paracellular mechanism-of-action increased the mucosal permeability of two peptide drugs, enalaprilat and hexarelin. This is in contrast to a previous SPIP study in which transcellular permeation enhancers only increased the small intestinal permeability of enalaprilat. This suggests that paracellular enhancers may be more effective for oral delivery of smaller peptides when used in conjunction with novel, absorption-enhancing drug delivery systems. Further, mucosal blood-to-lumen transport of ^{51}Cr-EDTA increased with the two paracellular permeation enhancers and with luminal hypotonicity. However, luminal hypotonicity did not affect the lumen-to-blood transport of enalaprilat and hexarelin. This suggests that hypotonicity affects paracellular solute transport only in the mucosal crypts, a mucosal area not accessible to luminal contents due to a constant, outward flow of water.

Author contributions

David Dahlgren, Markus Sjöblom, Hans Lennernäs designed the research. David Dahlgren, Tobias Olander, Markus Sjöblom, Mikael Hedeland, Hans Lennernäs carried out the experiments and performed data analysis. David Dahlgren, Tobias Olander, Markus Sjöblom, Mikael Hedeland, Hans Lennernäs participated part of the experiments. Mikael Hedeland provided quality control. David Dahlgren and Hans Lennernäs wrote the manuscript. David Dahlgren, Tobias Olander, Markus Sjöblom, Mikael Hedeland, Hans Lennernäs revised the manuscript. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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