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Invited Review

Intestinal absorption of small peptides: a review

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Summary Peptides display diverse structural features because of their varied amino acid compositions. The structural diversity often imparts them complex physiological functions, or possible health-beneficial effects. Some small peptides (di-/tripeptides) exert preventive effects against conditions such as hypertension, hypercholesterolaemia and atherosclerosis. Despite their health benefits, a limited understanding of peptide absorption may hinder their extensive application. Therefore, this review briefly introduces the *in vitro* and *in vivo* findings on the intestinal absorption of small peptides and potential factors affecting their absorption.

Keywords Absorption, intestinal membrane, PepT1, Peptide.

Introduction

Peptides are derived from food proteins through gastrointestinal digestion, or during food processing procedures such as fermentation or controlled protease hydrolysis, etc. In previous reports, we have demonstrated the health benefits of sardine muscle hydrolysate in modulating high blood pressure in patients suffering from hypertension. High blood pressure is caused due to degraded vessel function (contraction/relaxation), which leads to increased vessel resistance. We identified several small peptides (di-/tripeptides) such as Val-Tyr and Trp-His, etc., from sardine muscle hydrolysate as the functional component and the model of action of such antihypertensive small peptides is thought to result from the retardation of angiotensin I-converting enzyme (ACE) in the renin–angiotensin–aldosterone (RAA) systems. Alternatively, a series of our *ex vivo* studies have demonstrated that some small peptides derived from sardine muscle hydrolysate (Val-Tyr, Ile-Tyr, Tyr-Val and Trp-His) could induce relaxation of contracted aorta (Tanaka *et al*., 2006, 2009), and potentially lead to the prevention of atherosclerosis (Matsui *et al*., 2010). In the meantime, excessive studies on small peptides derived from food resources such as egg (Ile-Arg-Trp, Ile-Gln-Trp), fermented milk (Val-Pro-Pro, Ile-Pro-Pro and Leu-Pro-Pro) and soybean (Val-Pro-Tyr), etc. have been conducted, and the underlying mechanisms of the antihypertensive, anti-inflammatory, antidiabetic, kidney function ameliorative activities, etc. have been reported and reviewed (Kovacs-Nolan *et al*., 2012; Dias *et al*., 2014; Soga *et al*., 2014; Majumder *et al*., 2016; Iwaniak *et al*., 2018; Miralles *et al*., 2018). The physiological effects of bioactive peptides usually involve direct onsite interaction between the peptides and the targeted tissues and organs, that is, upon oral administration, the peptides have to be absorbed into the blood circulation in their active forms through the intestinal barrier. It is essential to study the absorption behaviour of such bioactive peptides in order to validate their bioactivities (Foltz *et al*., 2010; Matsui, 2018). Hence, further peptide research will be useful based on this review, in which the recent findings of small peptide absorption are summarised.

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In vitro absorption of small peptides

Fei *et al*. (1994) pioneered in cloning cDNA of rabbit intestinal peptide transporter, PepT1. The peptide binding cavity of PepT1 is reported to be approximately 13 × 12 × 11 Å, which limits the attachment to only di- and tripeptides, while would be sterically restrictive for peptides with >tetrapeptide length (Newstead *et al*., 2011). However, PepT1 is involved in the transport of several larger peptide prodrug molecules, such as valacyclovir (Brandsch, 2013). Therefore, it remains unclear if PepT1 could be used to transport oligopeptides (>tetrapeptides). Although other peptide transporters such as PHT1/2 (peptide-histidine transporters) and POT (oligopeptide transporters) are also expressed in the small intestine, PepT1 is reported to

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be mainly involved in the transport of small peptides, as it is expressed in a comparatively larger amount at the jejunal region (Herrera-Ruiz *et al*., 2001). It has been reported that the PepT1-mediated transport is driven by a proton gradient at the brush-border membrane (Ganapathy & Leibach, 1983). PepT1 has a characteristic twelve transmembrane domain composed of 710 amino acid residues (Fei *et al*., 1994). However, the peptide recognition site of PepT1 still remains unidentified (Meredith & Price, 2006).

The Caco-2 cell model, derived from human colon carcinoma cells, is a well-documented and widely used model to study the *in vitro* transport behaviour of target compounds (or penetrants). Differentiated Caco-2 cells express transporter proteins, paracellular junctions and digestive enzymes similar to those of the intestinal epithelial cells (Hidalgo *et al*., 2011). Caco-2 cells cultured on transwell inserts are cultured until they form monolayers with a transepithelial electrical resistance (TEER) of 400 Ω cm^2 or greater (Fig. 1). The food origin and *in vitro* Caco-2 cell transport behaviour of reported di-/tripeptides are summarised in Table 1. The transport behaviour of oligopeptides (>tetrapeptide) is not discussed in this review, as there are few evidence on *in vivo* absorption of intact oligopeptides (Matsui, 2018). Apparent permeability of P_{app} is shown in Table 1, as an indicator of the transport ability of penetrants across the Caco-2 cell membrane. Among them, Gly-*N*-methyl-Gly (Gly-Sar) showed the highest permeability of 38.6×10^{-6} cm s^{-1}. It was demonstrated that the presence of *N*-methylated peptide bonds results in high resistance against enzymatic degradation. Therefore, P_{app} values of peptides containing sarcosine may serve as a standard for comparative evaluation of the transport abilities of peptides (Hong *et al*., 2016). A preferential PepT1-mediated transport of small peptides with protease resistance was also confirmed by Ala-*N*-methyl-Phe-Ala, showing a P_{app} value of 2.5×10^{-6} cm s^{-1} (Gao *et al*., 2001), which was comparable to Gly-Sar-Sar (3.5×10^{-6} cm s^{-1}, Hong *et al*., 2016). The Caco-2 cell permeability of dipeptides is expected to be 10-fold higher than tripeptides according to the P_{app} values of Gly-Sar (38.6×10^{-6} cm s^{-1}) and Gly-Sar-Sar (3.5×10^{-6} cm s^{-1}), suggesting that the peptide chain length (size) could be important for membrane permeability. No correlation was observed between the log *P* and P_{app} values, indicating that the hydrophobicity of the peptides may not be a determining factor for their peptide transport ability. However, peptides of the same amino acid composition but present in a different order exhibited totally different permeability patterns (Trp-His vs. His-Trp, Ile-Phe vs. Phe-Ile), suggesting that the peptide sequence might affect its membrane permeability. It has been reported that the residues Glu26, Trp294 and Tyr588 in PepT1 are associated with substrate recognition (affinity), suggesting that aromatic or basic amino acid residue may play a key role in PepT1 recognition (Meredith & Price, 2006). However, *in silico* analysis using tripeptidyl thyrotropin-releasing hormone analogues revealed the difficulty of quantitatively predicting peptide transport, since the affinity of PepT1 to peptides can be determined by their diverse chemical interactions (Bagul *et al*., 2014). For example, a lower PepT1 affinity for the $_D$-amino acid-coupled peptidyl drug, acyclovir, compared to $_L$-amino acid-coupled drug indicated that stereoisomerism must also be considered as a determining factor of PepT1 recognition (Talluri *et al*., 2008).

In vivo absorption of small peptides

According to our first report on absorption of Val-Tyr in human blood (Matsui *et al*., 1999), *in vivo* absorption of small peptides seems to be as low as pmol or greater in blood. This indicates that analytical methods with high sensitivity and selectivity are needed for *in vivo*

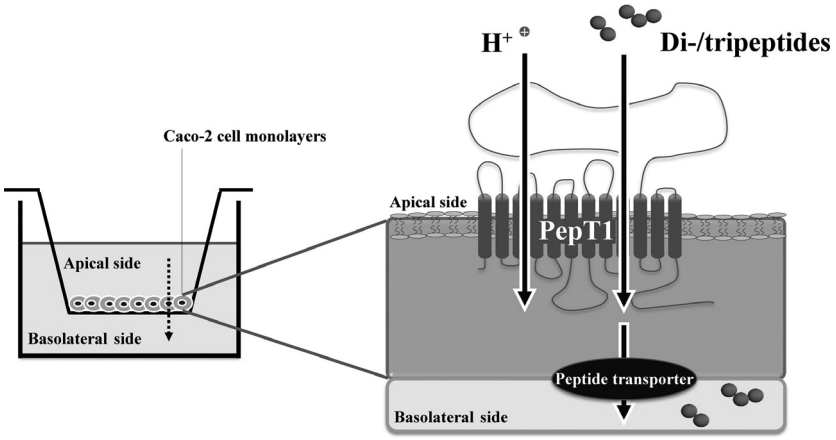

Figure 1 *In vitro* transport study of small peptides using Caco-2 cells differentiated on transwell semipermeable membranes. Peptide solutions are added to the apical side and samplings are performed in the basolateral side at indicated times to quantify the transported amount (usually using HPLC or LC-MS). PepT1 is a 12-transmembrane spanning protein. Di-/tripeptides are incorporated into the epithelial cells via PepT1 and released to the basolateral solution via a basolateral peptide transporter system. PepT1-mediated transport requires H^+ gradient as a driving force.

Table 1 Reported P_{app} of small peptides across Caco-2 cell monolayers

Chain length	Sequence	Origin	log *P*	P_{app} ($\times 10^{-6}$ cm s^{-1})	Assay	References
2	Gly-Sar	*Synthetic*	−1.65	38.6 ± 11.4	LC-MS	Hong *et al.* (2016)
2	Trp-His	Sardine	−0.56	8.1 ± 0.8	LC-MS	Tanaka *et al.* (2015)
2	His-Trp	Sardine	−0.62	4.6 ± 1.5	LC-MS	Tanaka *et al.* (2015)
2	Val-Tyr	Sardine	−0.76	6.8 ± 0.7	FL-HPLC	Takeda *et al.* (2013)
2	Ile-Phe	Soy	0.405	2.9 ± 0.2	FL-HPLC	Takeda *et al.* (2013)
2	Ala-Phe	Soy	−0.97	1.4 ± 0.2	UV-HPLC	Zhu *et al.* (2008)
2	Phe-Ile	Soy	1.667	1.4 ± 0.5	UV-HPLC	Zhu *et al.* (2008)
2	Tyr-Leu	Milk	1.014	3.28 ± 0.74	UV-HPLC	Osborne *et al.* (2014)
2	Leu-Tyr	Rapeseed	−0.25	3.49	LC-MS	Yang *et al.* (2017)
2	Thr-Phe	Rapeseed	−1.36	0.94	LC-MS	Yang *et al.* (2017)
3	Gly-Sar-Sar	*Synthetic*	−2.25	3.5 ± 0.3	LC-MS	Hong *et al.* (2016)
3	Leu-Lys-Pro	Egg	0.979	0.18 ± 0.01	LC-MS	Xu *et al.* (2017)
3	Ile-Gln-Trp	Egg	0.193	0.13 ± 0.01	LC-MS	Xu *et al.* (2017)
3	Leu-Ser-Trp	Soy	0.547	0.11 ± 0.01	LC-MS	Lin *et al.* (2017)
3	Ile-Pro-Pro	Milk	1.078	0.01 ± 0.009	LC-MS	Foltz *et al.* (2008)
3	Val-Pro-Pro	Milk	0.569	0.0005 ± 0.001	LC-MS	Foltz *et al.* (2008)

absorption studies of small peptides. Our first attempt to determine the absorbed peptides in blood was the application of naphthalene-2,3-dicarboxaldehyde (NDA) as a fluorescent reagent for peptide derivatisation (Matsui *et al.*, 1999). The technique using column-switching HPLC method with NDA fluorescence allowed the detection of the absorbed peptide in blood at pmol mL^{-1} levels, in which a maximum concentration (C_{max}) of 1.9 pmol mL^{-1}-plasma was observed in healthy individuals after 2-h of Val-Tyr (12 mg kg^{-1}) administration (Matsui *et al.*, 1999). The total absorption amount calculated using the plasma concentration-time curve per 1 mg dose (AUC/mg-dose) was calculated as 0.77 pmol h mL^{-1} mg^{-1}-dose. It showed about 1/70-fold lower absorption than that of a PepT1-transported dipeptidyl antihypertensive drug, captopril (53 pmol h mL^{-1} mg^{-1}-dose, Kripalani *et al.*, 1980). Although the proposed technique successfully explained about peptide bioavailability and its accumulation in organs (Matsui *et al.*, 2004), successive monitoring of ingested peptides in circulatory blood after administration of the same organism was difficult, since the assay required high volumes of blood (>1 mL). This method could not distinguish the ingested peptides from the endogenous metabolic peptides. Considering these disadvantages of conventional HPLC in combination with the derivatisation technique, current analytical assays for peptide absorption studies are mainly performed using mass spectrometry (MS). MS detection with liquid chromatographic (LC) separation (LC-MS) is a highly selective and sensitive assay, which can be used to quantify absorbed peptides in a small volume of plasma sample (>10 μL). Moreover, the use of isotope-labelled peptides allowed for selective analysis of absorbed peptides against the endogenous peptides (Nakashima *et al.*, 2011; Fig. 2).

Table 2 summarises the *in vivo* absorption profiles of di-/tripeptides including synthetic Gly-Sar peptides in rats. The extent of absorption ($AUC_{0–90\ min}$) of Gly-Sar and Gly-Sar-Sar were 569.83 ± 68.5 pmol h mL^{-1} mg^{-1}-dose and 430 ± 53.5 pmol h mL^{-1} mg^{-1}-dose, respectively. Though data were not shown, *in vivo* absorption of peptides were shown to be in a peptide-length-dependent manner (up to pentapeptides, Hanh *et al.*, 2017), which correlated with the *in vitro* findings that chain lengths could be a determining factor for peptide absorption. Besides in the synthetic peptides with *N*-methylated peptide bonds, the C_{max} of di-/tripeptides was at pmol order in rat blood. Among the peptides mentioned in Table 2, Trp-His showed the highest absorption with an AUC of 7.13 ± 1.87 pmol h mL^{-1} mg^{-1}-dose. However, considering the absorption of the therapeutic dipeptidyl drug, captopril, which was 53.0 ± 4.1 pmol h mL^{-1} mg^{-1}-dose and 10-fold higher than that of Trp-His, it must be noted that the *in vivo* intestinal absorption potential of natural small peptides is lower compared to pharmaceuticals. The absorption of dipeptides decreased in the following order of AUC: Trp-His > His-Trp > Val-Tyr > Leu-Tyr > Met-Tyr (Table 2), and it did not match the *in vitro* evaluation mentioned in Table 1 (P_{app}; Trp-His > Val-Tyr > His-Trp). This indicates that *in vitro* transportability of small peptides as P_{app} may not reflect *in vivo* absorption.

Factors affecting the absorption of small peptides

Current studies on the absorption of peptides are mainly conducted by a single oral administration of target peptides. However, as a food material, it is possible that co-existing compounds present in a food

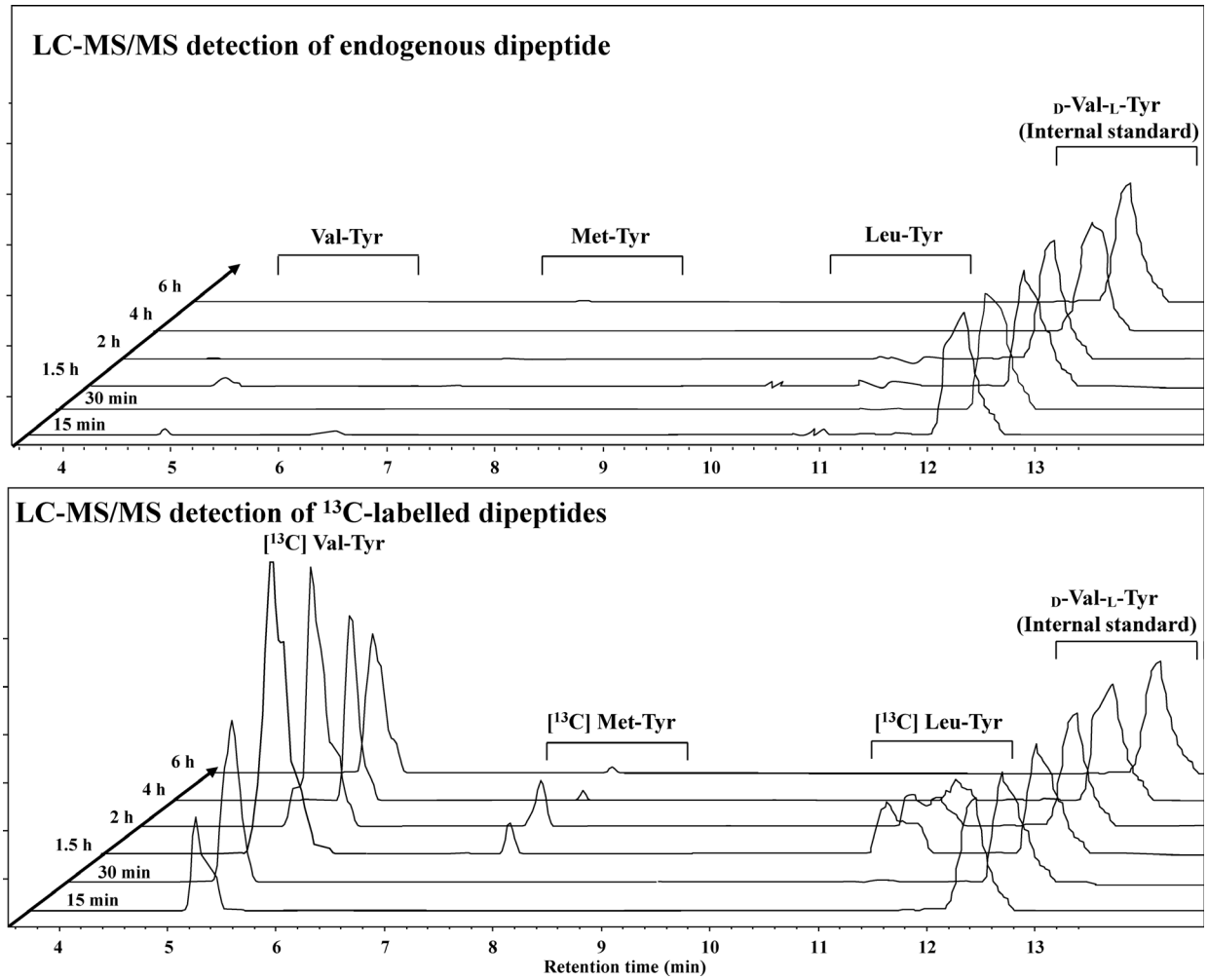

Figure 2 LC-MS stacked time-intensity chromatograms of peptides in plasma samples. Tandem MS detection enables highly sensitive and selective monitoring of endogenous dipeptides and ^{13}C-labelled dipeptides administered to rats in plasma samples (50 μL per analysis) successively from 15 min to 24 h after administration.

matrix could affect the absorption of peptides. For instance, although PepT1 exhibits a certain level of transport capacity and dose dependency (Jappar *et al*., 2011), the absorption of targeted small peptides could be competitively restricted when co-existing compounds share the same PepT1 transport pathway. In previous studies, we demonstrated that the transport of dipeptides Val-Tyr (Hong *et al*., 2013) and Trp-His (Tanaka *et al*., 2015) was inhibited by the existence of a PepT1 substrate Gly-Sar, using an *ex vivo* chamber transport experiment. Also, the blood concentration of a PepT1-transported dipeptidyl drug, captopril significantly decreased when administered simultaneously with a dipeptide Val-Tyr (Matsui *et al*., 2006). In such cases, the transport of dipeptide(s) mainly depends on the affinity (K_m) to PepT1. On the contrary, some food compounds could alter the absorption of dipeptides by adjusting the expression of PepT1. It has been reported that dietary amino acids and protein (hydrolysates) could up-regulate the expression of intestinal PepT1 mRNA to increase the intestinal transporter capacity of PepT1 and facilitate the absorption of peptides (Shiraga *et al*., 1999; Osmanyan *et al*., 2018). Meanwhile, theaflavins, polymeric catechins, have been reported to down-regulate the expression of PepT1 via the activation of intracellular AMP-dependent kinase, thus restricting the transport of dipeptides (Takeda *et al*., 2013). In addition, Wenzel *et al*. (2001) examined thirty-three food derived flavonoids and reported that quercetin, genistein, naringin, diosmin, acacetin and chrysin had the potential to increase the absorption of PepT1-transported compounds by activation of

Table 2 Reported peptide absorption in rats

Chain length	Sequence	C_{max} (pmol mL^{-1}-plasma)	t_{max} (h)	AUC (pmol h mL^{-1} mg^{-1}-dose)	$t_{1/2}$ (h)	References
2	Gly-Sar	12 200 ± 1800	0.1	569.8 ± 68.5	0.45	Hanh *et al.* (2017)
2	Trp-His	28.7 ± 8.9	1	7.13 ± 1.87	2.8	Tanaka *et al.* (2015)
2	His-Trp	1.1	1	1.4	1.9	Tanaka *et al.* (2015)
2	Val-Tyr	4.11 ± 1.13	1.5	0.64 ± 0.01	4.1	Nakashima *et al.* (2011)
2	Met-Tyr	0.38 ± 0.09	2	0.04 ± 0.002	4.1	Nakashima *et al.* (2011)
2	Leu-Tyr	0.54 ± 0.20	1.5	0.05 ± 0.002	4.1	Nakashima *et al.* (2011)
3	Gly-Sar-Sar	4600 ± 600	0.5	430 ± 53.5	1.25	Hanh *et al.* (2017)
3	Ile-Pro-Pro	12 ± 3	0.14	n.a.	0.16	Ten Have *et al.* (2015)
3	Leu-Pro-Pro	11 ± 3	0.12	n.a.	0.25	Ten Have *et al.* (2015)
3	Val-Pro-Pro	9 ± 2	0.15	n.a.	0.2	Ten Have *et al.* (2015)
–	Captopril	3700 ± 350	0.9	53.0 ± 4.1	n.a.	Kripalani *et al.* (1980)

n.a., not available.

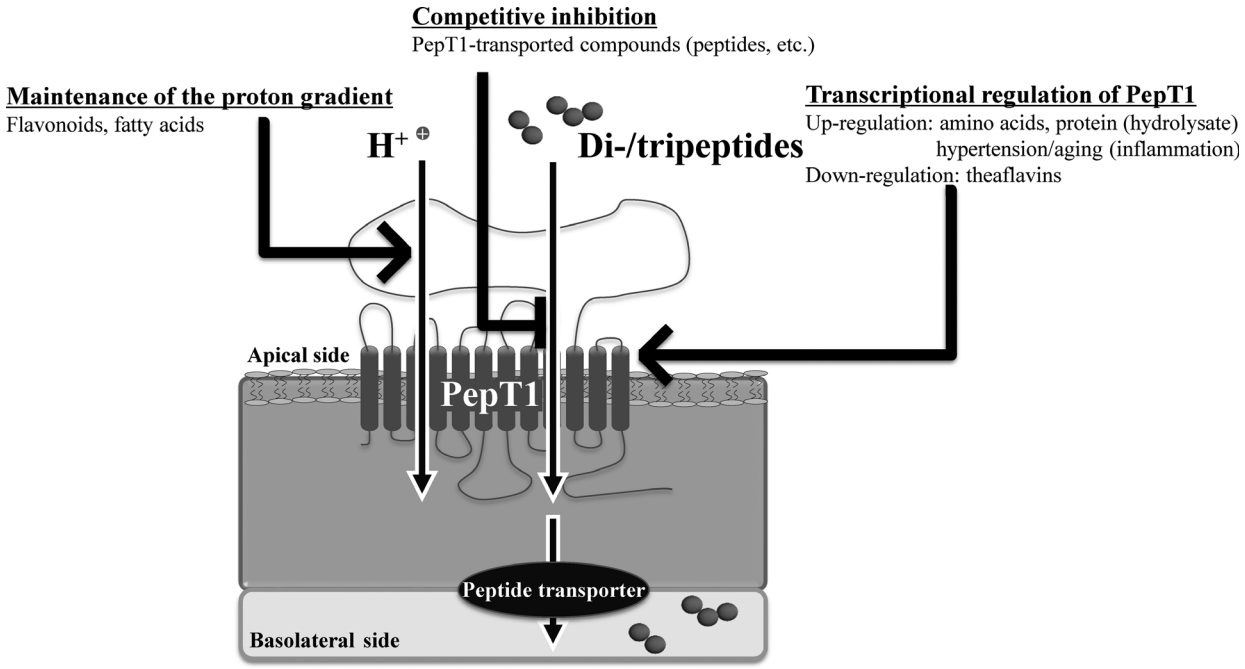

Figure 3 Factors affecting the absorption of small peptides. Food and non-food factors could affect the absorption of small peptides potentially via (1) competitive inhibition of PepT1, (2) transcriptional regulation of PepT1 and (3) maintenance of the proton gradient (a driving force for PepT1-mediated transport).

apical Na^+/H^+-exchanger to maintain the proton gradient which serves as a driving force for PepT1-mediated transport. Similarly, because a proton is released when a non-charged fatty acid enters the cytosol, dietary fatty acids could also facilitate the absorption of dipeptides by maintaining the transmembrane proton flux (Spanier *et al.*, 2009; Fig. 3). With regards to other macronutrients, carbohydrates have been reported to stimulate intestinal tissue anabolism, thus incorporating more peptides into the intestinal cells than released into blood circulation. In contrast, addition of fibre increased the systemic availability of di-/tripeptides (Ten Have *et al.*, 2015). However, information is still limited on the effects of respective macronutrients on the absorption of small peptides.

Besides food factors, the absorption of small peptides could also be affected by other factors such as health of the individual. In a recent study, we reported enhanced absorption of dipeptides Gly-Sar and Trp-His in 40-week-old spontaneously hypertensive rats

(SHRs) and compared them to 8-week young SHRs. We discovered that the expression of intestinal PepT1 was significantly (~1.5-fold) higher in the 40-week SHRs, which could indicate its enhanced absorption (Hanh *et al*., 2017; Fig. 3). Although the mechanisms underlying the elevated expression of PepT1 are still not clear, we speculated that chronic inflammation and hypertension in ageing individuals could be involved in the regulation of intestinal PepT1 (Ingersoll *et al*., 2012).

Conclusion

This review summarises the current understanding on the absorption of small peptides. In future studies, when evaluating the absorption of di-/tripeptides, or even longer peptides, assay methods with high sensitivity and selectivity should be used due to the low concentration level of peptides in blood. Moreover, the impact of both food and non-food factors on the absorption of small peptides should also be taken into consideration.

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Conflict of interest

There are no conflicts to declare.

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