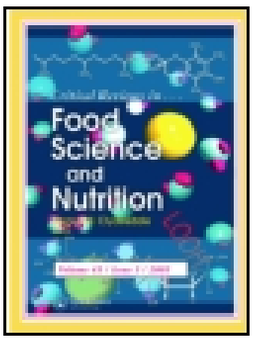

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Effects of natural peptides from food proteins on angiotensin converting enzyme activity and hypertension

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ABSTRACT

Cardiovascular diseases are the leading cause of death. The underlying pathophysiology is largely contributed by an overactivation of the renin-angiotensin-aldosterone-system (RAAS). Herein, angiotensin II (AngII) is a key mediator not only in blood pressure control and vascular tone regulation, but also involved in inflammation, endothelial dysfunction, atherosclerosis, hypertension and congestive heart failure. Since more than three decades suppression of AngII generation by inhibition of the angiotensin-converting enzyme (ACE) or blockade of the AngII-receptor has shown clinical benefit by reducing hypertension, atherosclerosis and other inflammation-associated cardiovascular diseases. Besides pharmaceutical ACE-inhibitors some natural peptides derived from food proteins reduce *in vitro* ACE activity. Several animal studies and a few human clinical trials have shown antihypertensive effects of such peptides, which might be attractive as food additives to prevent age-related RAAS activation. However, their inhibitory potency on *in vitro* ACE activity does not always correlate with an antihypertensive impact. While some peptides with high inhibitory activity on ACE-activity *in vitro* show no antihypertensive effect *in vivo*, other peptides with only a moderate ACE inhibitory activity *in vitro* cause such effects. The explanation for this conflicting phenomenon between inhibitory activity and antihypertensive effect remains unclear to date. This review shall critically address the effects of natural peptides derived from different food proteins on the cardiovascular system and the possible underlying mechanisms. A central aspect will be to point to conceptual gaps in the current understanding of the action of these peptides with respect to *in vivo* blood pressure lowering effects.

KEYWORDS

Bioactive peptides; antihypertensive peptides; hypertension; Angiotensin Converting Enzyme; Cardiovascular disease

1. Introduction

According to the World Health Organization (WHO), cardiovascular diseases (CVD) such as atherosclerosis, coronary heart disease, stroke and heart failure rank highest in the cause of global death (World Health Organization (WHO), 2014). Arterial hypertension is one of the most important risk factors of these diseases (Borghi and Rossi, 2015). One of the key players that regulate blood pressure and vascular tone is the renin angiotensin system (RAS). Renin, a glycoprotein synthesized from the juxtaglomerular cells of the renal afferent arteriole, is secreted into the circulation and cleaves liver-derived angiotensinogen to form the decapeptide angiotensin I (AngI) (Griendling et al., 1993). This inactive decapeptide is hydrolyzed by the angiotensin converting enzyme (ACE) into the strong vasoconstrictor angiotensin II (AngII). Additionally, ACE metabolizes bradykinin (BK), a vasodilator, to inactive BK-(1–7) and BK-(1–5) (Figure 1) (Carey and Siragy, 2003). Hence ACE has a dual direct role in the vasculature in that it promotes the production of Ang II, a potent vasoconstrictor while degrading BK, a vasodilator. Effects of Ang II are mainly mediated via the G protein coupled angiotensin type 1 and type 2 (AT1 and AT2) receptors. Most of the Ang II effects are mediated through AT1 receptors causing vasoconstriction, aldosterone secretion, renal tubular Na^+ reabsorption, sympathetic nerve activation, cardiac inotropic and chronotropic actions and cardiovascular inflammation, hypertrophy and fibrosis (Nguyen Dinh Cat and Touyz, 2011). The AT2 receptor elicits effects, which counteract those of the AT1 receptor, but the impact of AT2 activation is as yet less clear. This receptor is the predominant Ang II receptor in the fetus, but it is expressed only at low levels in the adult vasculature, juxtaglomerular cells, glomeruli and tubules and studies of the importance of this receptor are controversial (Horiuchi et al., 1999). However, increased AT2 receptor expression occurs under pathological conditions, such as hypertension, myocardial infarction, cardiac and renal failure, cerebral ischemia and diabetes (Abadir et al., 2011; Savoia et al., 2005). Interestingly our goup recently demonstrated a higher expression of AT2 receptors in female rats as compared with male rats of the same age. This effect was age-dependent and associated with a lower Ang II dependent vasoconstriction in female as compared with male vessels (Al-Gburi et al., 2017). Beside the classical angiotensins further peptides, e.g. angiotensin 1–7 (Ang 1–7), seem to be involved in regulation of the RAS (Figure 1). Ang 1–7 is produced from Ang I or Ang II by the catalytic activity of angiotensin-converting enzyme 2 (ACE2) and ACE. This peptide causes vasodilatation, anti-proliferative effects and natriuresis and activates the bradykinin-NO pathway (Santos et al., 2013). Hence,

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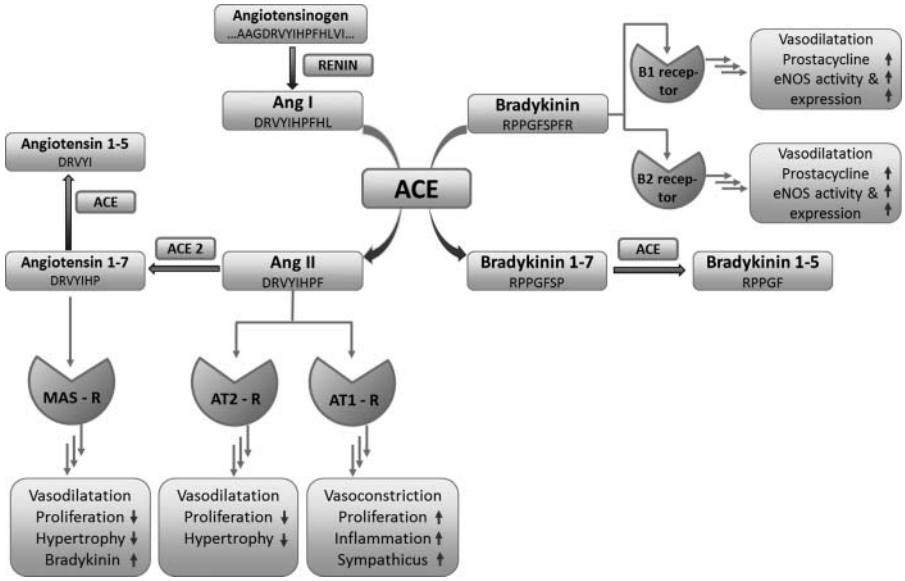

Figure 1. Scheme of the renin angiotensin system (RAS), highlighting the role of angiotensin converting enzyme (ACE) and related physiological actions; Ang, angiotensin; AT1-R, Ang II type 1 receptor; AT2-R, Ang II type 2 receptor; MAS-R, mas G-protein coupled receptor.

Ang 1–7 antagonizes the pressor effect of AT1-receptor stimulation via Ang II, suggesting that it may act as a brake on AT1-receptor agonist dependent effects resulting in organ protective effects, e.g. reduction of cardiac hypertrophy and fibrosis as well as renal damage. It is thought that under pathophysiolocial conditions this complex balance in the RAS system is disturbed. Hence, inhibition of the RAS is pursued and nowadays represents the first line treatment in clinical routine (Fernandez-Musoles et al., 2013). The impact of RAS inhibitors, like ACE inhibitors or AT1-receptor blockers, was convincingly shown in several clinical trials, such as HOPE (Heart Outcomes Prevention Evaluation) and EUROPA (European trial on Reduction Of cardiac events with Perindopril in stable coronary Artery disease) demonstrating that ACE inhibitors reduce cardiovascular mortality and morbidity in patients with established coronary artery disease without left ventricular dysfunction (Fox, 2003; Yusuf et al., 2000). Despite these positive effects of RAS inhibitors, especially ACE inhibitors exhibit unwanted side effects, which include hypotension, electrolyte imbalance, angioedema and dry cough (Pfeffer et al., 2003). This is a major disadvantage in the treatment of prehypertensive patients, who exhibit a chronic systolic blood pressure elevation to values between 120 and 139 mm Hg and diastolic blood pressure between 80 and 89 mm Hg. These patients are very prone to develop hypertension subsequently (Chobanian et al., 2003). However, because of mentioned side effects, prehypertensive patients do usually not start any pharmacological treatment as long as there are no further cardiovascular risks or vascular damages (Collier and Landram, 2012). This happens despite of evidence that prehypertensive patients without further cardiovascular risks could benefit from the early onset of treatments (Jung et al., 2017; Tao et al., 2017). (Pre)hypertensive patients, with or without drug treatment, are generally advised to change their current lifestyle, including measures to decrease overweight, to adopt a healthy dietary habit and lower sodium intake and to engage in a more active lifestyle. In this context alternative dietary approaches aiming to lower the age dependent rise in arterial blood pressure have received a broader attention during the last decade. It is of particlur interest that some natural peptides from food proteins have structural similarities with pharmaceutical ACE-inhibitors and show ACE inhibiting potential (Korhonen and Pihlanto, 2006). As hypertension is a significant public health problem worldwide (World Health Organization (WHO), 2014), these ACE inhibitory peptides, as part of food products or as nutraceuticals, may be of functional interest for early treatment or disease delay for people suffering from mild hypertension. Biologically active peptides with a special reference to ACE-inhibition are food-derived peptides that in addition to their nutritional value exert an effect on ACE activity in the body. These peptides are usually inactive within the original protein, but once hydrolysed from the parent protein, they play a role as regulatory compounds with hormone-like activity that is based on the amino acid composition and sequence (Meisel, 1997). This review critically addresses the current evidence that natural peptides derived from different food proteins act on the cardiovascular system by exerting ACE inhibition. It is a central aim of this review to reveal conceptual gaps in the current understanding of the action of these peptides with respect on ACE activity and antihypertensive effect. In addition, future perspectives of these peptides are discussed. This review is based on a comprehensive and systematic literature search within 3 databases (pubmed/medline, Chochrane, National Library of Medicine) and google scholar. The search was conducted using the following keywords and combinations thereof: antihypertensive peptide, ace inhibitory peptide, hypertension, blood pressure, cardiovascular effects, vasodilation, antihypertensive effect, spontaneously hypertensive rat, human study, absorption, transport, Pept1, stability, bioavailability, *in vivo*, renin angiotensin system, ACE inhibitory peptide, dairy peptide, food-derived peptide, angiotensin converting enzyme, binding site, target site. In addition, the reference lists of included studies

were analyzed to find additional studies. Studies with poor study design, such as missing randomization in *in vivo* studies, and missing data in methods and unsubstantiated interpretations were excluded.

2. ACE-inhibitory effects of peptides *in vitro*

Up to date the knowledge of the structure – activity relationship of ACE- inhibiting peptides is limited. While a major part of current research focuses on the generation and identification of ACE inhibiting and antihypertensive peptides, little progress has been made on understanding the correlation between peptide sequence and its bioactivity as an ACE inhibitor. Descriptively, most potent ACE inhibitory peptides have short sequences, with typically 2–12 amino acids. This short sequence has been considered important in order to achieve an antihypertensive effect *in vivo*, because short peptides are likely better absorbed into the circulation and, if they are not hydrolytically modified, should retain their ACE-inhibitory effect. However, there are also examples of peptides with longer peptide length having an ACE inhibiting effect (Lee et al., 2010; Qian et al., 2007; Jakubczyk et al., 2013). In these cases the *in vitro* measured effect may not be relevant for the *in vivo* situation, because longer peptides are likely to be partially degraded by peptidases in the GI-tract.

The structure of the active site of the enzyme is crucial for an understanding of the inhibitor binding mechanism. ACE is a zinc-dependent membrane-bound dipeptidyl-carboxypeptidase accepting a relatively wide range of substrates (Bernstein et al., 2013). Two catalytically active isoforms of ACE have been identified, somatic and testicular ACE. The somatic ACE (sACE) is a protein with 1277 amino acids composed of a repeated structure of two homologous active sites, the so-called N- and C-domain (Soubrier et al., 1988). The testicular ACE (tACE) has only a single domain, which is identical to the C-domain, except for a 36-residue, serine- and threonine-rich sequence at the N-terminus of testis ACE (Ehlers et al., 1989). Gene expression analysis indicates that sACE mRNA is expressed in virtually all tissues and strongly expressed in endothelial cells, especially in the capillaries of the lung, as well as in epithelial cells of the kidney, small intestine and epididymis. In contrast, tACE is exclusively expressed in male germ cells (Coates, 2003). Both isoenzymes are released as soluble proteins after a specific proteolytic cleavage in the juxtamembrane stalk (Oppong and Hooper, 1993; Wei et al., 1991), catalysed by a class of proteases variously referred to as membrane-protein-solubilizing proteases, sheddases or secretases (Hooper et al., 1997). Somatic ACE is shedded between amino acid residues arginine1203 and serine1204 (R1203 and S1204, in the following the one letter code for amino acids – Table 1 – is used), but soluble ACE represents less than 2% of somatic ACE and does not seem to have an important physiological role (Levitt and Schoemaker, 2006). The C- and N-domain are structurally very similar and both contain the zinc-binding motif HEXXH (where X represents any amino acid). Both H residues are ligands of the zinc ion, the third being an E. The amino acid E in the HEXXH motif is critically involved in catalysis. It binds the water molecule, which initiates a nucleophilic attack on the peptide bond in the substrate (Hooper, 1994). Despite the structural similarities both domains have different substrate specificities (Bernstein et al., 2011). The physiologically most important substrates for ACE, AngI and bradykinin may be degraded by both domains. However, the C-domain hydrolyses AngI to a greater extent, whereas affinity to bradykinin is twotimes higher for the N-domain (Jaspard et al., 1993). Binding studies confirmed the higher impact of the C-domain for the AngII production (Fuchs et al., 2008; Georgiadis et al., 2003) and the influence on blood pressure (Fuchs et al., 2008). Other natural peptides, which are mainly hydrolysed by the N-domain, are Acetyl-SDKP, the luteinizing releasing hormone (LH-RH), Ang1–7, Angiotensin 1–9 and gonadotropin-releasing hormone (GnRH) (Rousseau et al., 1995; Junot et al., 2001; Ding et al., 2016; Anthony et al., 2012).

In gereral, the subsites S1, S1′, S2′ and the zinc-ion are responsible for the binding of substrate and competitive inhibitors, respectively (Figure 2). The affinity of a substrate or an inhibitor to the active center of ACE is mainly dependent upon the C-terminal amino acids. This is exemplified in Figure 2 for the well-known ACE inhibitors captopril and lisinopril. An X-H-group is located between S1′ and S2′ (X is V in the C-domain and S in the N-domain, respectively), which forms hydrogen bonds with the substrate or inhibitor. The positive charge (from the guanidino-group of Arg) beside the S2′ subsite may interact with the negatively charged oxygen at the C-terminus of the respective substrate. The zinc-ion, which is located between the S1 and S1′ subsites, is responsible for the hydrolytic cleavage of the substrate peptide bond. Effective inhibitors, such as captopril and lisinopril (Figure 2), have no peptide bound at this position, thus resulting in an effective blockade of the active center (Li et al., 2004). A strong interaction with the S1′ and S2′ subsites was determined for aromatic molecules (Cheung et al., 1980). This influence is also seen in Figure 2. While captopril binds selectively to S1′ and S2′, lisinopril also binds with the aromatic ring at the S1 subsite and, thus, a stronger binding results. Additionally, the presence of a K residue in the sequence of lisinopril enhances the binding to the S1′ subsite (Acharya et al., 2003). The stronger binding is reflected in a higher inhibitory action with an IC_{50} (concentration, which is necessary to inhibit enzyme activity 50%) of 0.7 nM for lisinopril as compared to 2 nM in the case of captopril (Michaud et al., 1997).

The difference of the two domains with respect to substrate specificity may be explained by structural differences related to the amino acid sequence. While in the S2′ subsite in the active center of the C-domain two V residues (V379 and V380) are present, they are exchanged in the N-domain with S (S357) and T (T358) residues, which are more hydrophilic. Therefore, a hydrophobic molecule, such as W, at the C-terminus could strongly interact with the two V residues in the S2′ subsite of the C-domain. In contrast, weaker interaction is expected with the more hydrophilic area of the N-domain (Kröger et al., 2009).

In general, classical synthetic ACE inhibitors have low specifity for one of the two domains of ACE. Only in the case of lisinopril a slightly higher affinity to the C-domain was reported (Michaud et al., 1997). Up to now only a few domain specific

Table 1. One letter amino acid code.

ONE LETTER CODE	AMINO ACID
A	alanine
R	arginine
N	asparagine
D	aspartic acid
C	cysteine
Q	glutamine
E	glutamic acid
G	glycine
H	histidine
I	isoleucine
L	leucine
K	lysine
M	methionine
F	phenylalanine
P	proline
S	serine
T	threonin
W	tryptophan
Y	tyrosine
V	valine
O	pyrrolysine
X	unknown

inhibitors are described in literature. Because of the dominant role in the production of AngII, C-domain specific inhibitors may be particularly attractive, such as the lisinopril analogue lisinopril-tryptophan S-enantiomer, the peptide RXPA380 and the ketomethylene peptide (5S)-5-[(N-benzoyl)-amino]-4-oxo-phenylhexanoyl-L-tryptophan (Watermeyer et al., 2008; Kröger et al., 2009). It is suggested that especially the bulky W residue is responsible for the C-domain selective inhibition (Watermeyer et al., 2008; Kröger et al., 2009). This supported by a recent study of Lunow et al., (2015), in which the C-domain selectivity of W-containing dipeptide inhibitors was investigated. Especially the peptides AW, VW, LW and IW had a high selectivity for C-domain inhibition (Lunow et al., 2015).

Studies on structure–activity correlations among peptide ACE inhibitors indicate that binding to ACE is strongly influenced by structural characteristics of the peptide, rather than by peptide length. Table 2 shows selected ACE inhibitory peptides contained in food proteins. Peptides are listed in the order of peptide length with information on the IC_{50} value and preferential protein sources, respectively. It has to be mentioned that the experimental conditions for ACE activity measurements reported in literature differ with respect to incubation times and the synthetic substrates used. However, for better comparison within this review ACE-activity of all selected inhibitor peptides listed in Table 2 was measured according to Cushman and Cheng (Cushman and Cheung, 1971) with slight modifications. Most frequently, the AngI analogue hippuryl-histidyl-leucine was used as substrate and the inhibitory effect was tested, as usual for *in vitro* screening, on the example of rabbit lung ACE.

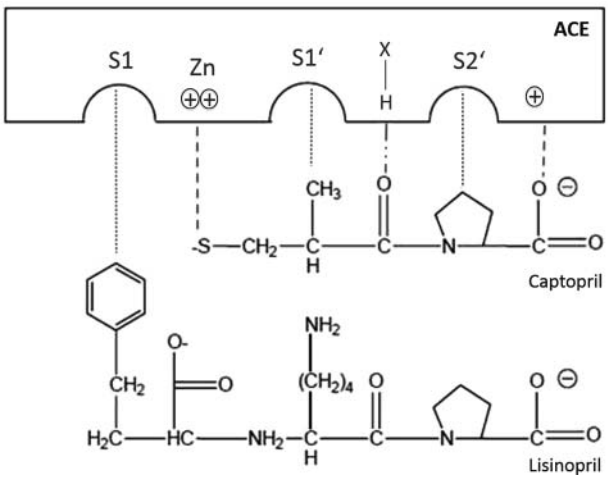

Figure 2. Binding model of the active center of ACE [Li et al., 2004]. S1, S1′, and S2′ represent side chain binding sites within the active sites of ACE. "+" indicates positive charges and X-H represents a potential hydrogen bond. Interactions stabilizing substrates or inhibitors are indicated by broken and dotted lines. Inhibitor binding is shown on the example of two ACE inhibitors, captopril and lisinopril.

The peptide structure is important for the effective binding in the active center of ACE. Hydrophobic and bulky amino acid residues (aromatic or branched amino acids) at the C-terminal position of the peptides support binding to ACE. Especially the presence of hydrophobic P residues at the C- terminal position seems to enhance the ACE inhibitory potency of peptides. Other aromatic residues as Y, F and W are also present at the C-terminus of many potent peptide ACE inhibitors, especially in di- and tripeptide inhibitors (Cheung et al., 1980; Meisel et al., 2006; Table 2). Furthermore, the positive charge on the side chains of R and K residues at the C-terminus has been noted to contribute to the ACE inhibitory potential (Meisel et al., 2006; Ondetti et al., 1977). While the binding is weak if P is present at the N-terminal site, V or I at this position offer a good binding to ACE.

Most peptides with an ACE inhibitory effect act as competitive inhibitors. Here the interaction between the peptide C-terminus and the ACE subsites S1, S1′, and S2′ determines the binding strength in the active center. As seen in table 2, bulky aromatic residues, which have a high affinity to the S1, S1′ and S2′ subsites cause a strong inhibitory effect (Wu et al., 2006a). This result is supported by in silico studies, which indicate that in the case of dipeptides amino acid residues with bulky side chains or hydrophobic side chains should provide superior ACE inhibition. For tripeptides, the most favorable residues for the carboxyl terminus were P and aromatic amino acids, while positively charged amino acids were preferred for the middle position and hydrophobic amino acids for the N-terminus (Wu et al., 2006a). But P at the C-terminus is not generally best for ACE inhibitory effects. In peptides of 4–10 amino acids length the C-terminal tetrapeptide residues seem more important for their ACE-inhibitory activity than the C-terminal tripeptide residues. The most promising amino acid residues starting from C-terminus are Y and C for the first position, H, W and M for the second position, I, L, V for the third, M and W for the fourth position (Wu et al., 2006b).

Naturally occurring peptides with ACE inhibitory effect were first detected in snake venom (Ferreira et al., 1970; Kato and Suzuki, 1971; Ondetti et al., 1971). Most of these ACE inhibitor peptides had a C-terminal sequence of AP or PP in the nonapeptide e.g. OWPRPQIPP. The IC_{50} value was determined in the order of 0.9 μM and the peptides were shown to effectively lower blood pressure *in vivo* (Engel et al., 1973; Ondetti et al., 1971). The production of structurally related

Table 2. ACE inhibitory peptides from food proteins.

Source	Peptide sequence	IC_{50}* [μM]	Reference
Corn gluten	AY	14.2	Yang et al., 2007
Alpha – casein	FY	3.74	Suetsuna, 1998
Wheat	IY	5.3	Rudolph et al., 2017
Beta-lactoglobulin,	VY	26.0	Martin et al., 2008
Salmon muscle	YP	5.21	Neves et al., 2017
Beta Casein	YG		FritzGerald & Meisel, 2000
Shark	VW	3.3	Ikeda et al., 2015
Wakame	AW	15.4	Sato et al., 2002
Alpha lactalbumin	IW	0.7	Martin et al., 2008
	WL	10.0	
Whey	KW	4.3	Rudolph et al., 2017
Pea, Rice, Soy	FW	9.5	
Egg (ovalbumin)	LW	6.8	Fujita et al., 2000
Soy	WW	81.3	Wu et al., 2006
Soy	AF	165.3	Zuh et al., 2008
	IF	65.8	
Shark	FE	1.45	Wu et al., 2008a
Shrimps	DP	2.15	Wang et al., 2008
Salmon	FL	13.6	Enari et al. 2008
Beta casein	IPP	5.0	Nakamura et al., 1995
	VPP	9.0	
Buckwheat	GPP	6.25	Ma et al., 2006
Bonito	LKP	0.32	Fujita & Yoshikawa, 1999
Bonito	LRP	1.0	Matsumura et al., 1993
Chicken	LAP	3.5	Fujita et al., 2000
Grass carp	VAP	5.34	Chen et al., 2012
Spirulina platen.	IQP	5.77	Lu et al., 2010
Bonito	ILP	1.7	Yokoyama et al., 1992
Wheat	IAP	2.7	Motoi et al., 2003
Rice	VWP	4.5	Chen et al., 2013
Lysozyme	VAW	2.86	Rao et al., 2012
Chicken	IKW	0.21	Fujita et al., 2000
Rubisco	MRW	0.6	Zhao et al. 2007
Bovine	GPL	2.55	Kim et al., 2001
Broccoli	YPK	10.5	Lee et al., 2006
Sesame	LKY	0.78	Nakano et al., 2006
Wheat germ	IVY	0.48	Matsui et al., 2000
Soybean	HHL	2.2	Shin et al., 2001
Sardine	AKK	3.13	Matsui et al., 1994
Zein	IRA	6.40	Miyoshi et al., 1991
Rice	TQVY	18.2	Li et al., 2007
Sake	YGGY	3.4	Sarro et al., 1994
Catfish	GPPP	0.86	Ghassem et al., 2012
Sardine	GWAP	3.86	Matsui et al., 1994
Spirulina platen.	IAPG	11.4	Suetsuna & Chen 2001
Wakame	YNKL	21.0	Suetsuna & Nakano 2000
	YKYY	64.2	
Soybean	VLIVP	1.69	Gouda et al., 2006
Bonito	LKPNM	2.4	Fujita & Yoshikawa, 1999
Lactoferrin	LIWKL	0.47	Ruiz-Gimenez et al., 2012
Egg	RVPSL	20	Liu et al., 2010
Bonito	ALPHA	10.0	Matsumura et al., 1993
Cuttlefish	VELTP	5.22	Balti et al., 2015
Spinach rubisco	LIRPVA	0.38	Yang et al., 2003
Soybean	NWGPLV	21	Motoi et al, 2003
Chicken	FQKPKR	14.0	Fujita et al., 2000
	NIFYCP	15.0	
	FKGRYYP	0.55	
Beta lactoglobulin	ALPMHIR	42.6	Mullally et al., 1997
Salmon	FNVPLYE	7.72	Ahn et al., 2012
Chicken	GAXGLXGP	29.0	Saiga et al., 2008
Soybean	LAIPVNKP	70.0	Kuba et al., 2005
Oyster	VVYPWTQRP	0.07	Wang et al., 2008
Walnut	WPERPPQIP	25.67	Liu et al., 2013
Boy jellyfish	ACPGPNPGRP	2.03	So et al., 2016
Algae	VECYGPNRPQF	29.6	Sheih et al., 2009
Bullfrog	GAAELPCSADWW	0.95	Qian et al., 2007b
Tuna	WPEAAELMMEVDP	21.6	Qian et al., 2007a
Ostrich egg	AFKDEDTEEVPFR	80.2	Tanzadehpanah et al., 2013
Squid gelatin	GPLGLLGFLGPLSAPGAP	90.3	Aleman et al., 2011

*IC_{50} = inhibitor concentration, which is necessary to inhibit 50% of enzyme activity.

synthetic ACE inhibitors was based on these studies. More recently, ACE inhibitory peptides encrypted in food proteins have been discovered (Oshima et al., 1979). Compared to the snake venom peptide OWPRPQIPP several peptides from various food proteins show a quantitatively similar inhibitory effect (Table 2). Animal sources are milk proteins, such as casein (Foltz et al., 2008; Gobbetti et al., 2000; Jiang et al. 2010; Kim and Chung, 1999; Kohmura et al., 1989; Muruyama et al., 1987; Muruyama and Suzuki, 1982; Pihlanto-Leppälä et al., 1998; Yamamoto et al., 1994; Xue et al., 2017) and whey protein (Abubakar et al., 1998; Hernandez-Ledesma et al., 2002; Martin et al., 2008; Mullally et al., 1997; Pihlanto-Leppälä et al., 2000; Pihlanto-Leppälä, 2001; Tauzin et al., 2002), fish (Astawan et al., 1995; Balti et al., 2010; Matsui et al., 1994; Matsui et al., 1993; Matsumura et al., 1993; Wako et al., 1996; Yokoyama et al., 1992), porcine and chicken muscle (Arihara et al., 2001; Fujita et al., 2000; Nakashima et al., 2002) and egg (Fujita et al., 2000; Majumder and Wu, 2010; Yoshii et al., 2001). Some typical representatives for plant sources are algae (Sato et al., 2002; Suetsuna and Chen, 2001; Suetsuna and Nakano, 2000; Suetsuna, 1998), soy (Gu and Wu, 2013; Shin et al., 2001; Shin et al., 1995; Wu and Ding, 2001), rice (Rudolph et al., 2017), wheat germ (Jia et al., 2010; Matsui et al., 2000; Matsui et al., 1999), corn gluten (Marutama et al., 1989; Miyoshi et al., 1991; Suh et al., 2003; Suh and Whang, 1999; Yano et al., 1996), bean proteins (Kwon et al., 2000; Leen et al., 1999), garlic (Sarro et al., 1994) and buckwheat protein (Li et al., 2002). Due to the broad range of possible ACE substrates, it is not surprising that several peptide sequences could lead to an inhibitory effect.

In summary, the diversity of food-derived peptides with ACE inhibitory activity is high. Also, among these studied peptides the inhibitory potential differs widely. Using the snake venom peptide OWPRPQIPP as a guiding compound with an *in vitro* IC_{50} around 0.9 μM one dipeptide (IW) and several tripeptides (LKP, MRW, IKW, LKY, IVY) have been identified to show IC_{50} values below 1 μM. To a smaller extent also tetra- to dodeca-peptides with ACE-inhibitory potential in this IC_{50} range have been reported (GPPP, LIWKL, LIRPVA, FKGRYYP, VVYPWTQRP, GAAELPCSADWW). Particularly di- and tripeptides show a high inhibitory activity. Less common, but still representative, are peptides with longer amino acid sequences. Binding in the target sites is supported by bulky and more hydrophobic amino acids, especially at the C-domain. Although a low IC_{50} value determined *in vitro* is a prerequisite for the inhibitory potential of a compound, further aspects e.g. the bioavailablility of natural peptides under *in vivo* conditions also need to be considered.

3. Peptide stability and bioavailability

When applied to living organisms peptides rapidly undergo enzymatic hydrolysis. With the aim to develop innovative food products or neutraceuticals only oral application is of interest. Once absorbed into the blood, peptides need sufficient stability to reach their site(s) of action. Therefore, peptide stability has at least to be considered for the gastrointestinal (GI) tract and blood plasma. In addition, epithelial transport in the gastrointestinal tract is of functional importance as well as elimination via excretion.

Peptides may undergo hydrolysis by digestive proteases in the intestinal lumen and can be subjected to further cleavage by several membrane-anchored epithelial cell peptidases. Some studies have been carried out to investigate the stability of ACE inhibitory peptides against GI digestion. The resistance toward breakdown is studied by simulating the GI process by sequential hydrolysis of ACE inhibitory peptides with pepsin, trypsin and chymotrypsin, respectively (Escudero et al., 2014; Garcia-Tejedor et al., 2014; Gomez-Ruiz et al., 2004; Hernandez-Ledesma et al., 2004; Matsui et al., 1999; Miguel et al., 2006; Tavares et al., 2011; Vermeirssen et al., 2003; Walsh et al., 2004). In their studies, Miguel et al., (2006) showed that peptides isolated from ovalbumin had different stability against simulated GI digestion. While small peptides, such as YPI, and peptides containing P at the C-terminus (RADHP and ADHP) were stable, slight changes in the structure, e.g. by adding one or two amino acids to the C-terminus (e.g. RADHPF, RADHPFL, FRADHPFL), caused a higher instability under simulated GI hydrolysis. The ACE inhibitor YGGY (IC_{50} = 3.4 μM) even lost its complete ACE inhibitory potential after simulated GI digestion (Saito et al., 1994). It has been noted that certain peptide structures are resistant to gastrointestinal enzymes due to the sequence and position of their amino acids within the peptides. Peptides containing P and hydroxyl-P residues, especially sequenced at the C-terminus, are unaffected by the digestive proteases (Jao et al., 2012). Foltz et al., (2009) investigated the relationship between dipeptide structure and dipeptide stability. While presence of F, Y and W residues at the C-terminus favors ACE inhibition, the stability of these aromatic amino acid residues against GI digestion is poor. In contrast peptides with the amino acids N, P, S and T at the C-terminus are considered as stable. Aliphatic side chains, such as V and I at the N-terminus have favourable impact on the stability against GI enzymes. A superior stability is achieved with P, D and G at the N-terminus, whereas A, R, C, L, K and M at this position have only poor stability (Foltz et al., 2009, Table 3). Furthermore, P, D and G at the N-terminus protect dipeptides during simulated intestinal digestion independent of the C-terminal amino acid residue (Foltz et al., 2009). It is noted, however, that GI enzymes not only degrade bioactive peptides, but there is also the potential by hydrolyzing an inactive peptide to generate peptides with a potent ACE inhibitory activity (Hernandez-Ledesma et al., 2004; Tavares et al., 2011). Along this line, the peptide IYPRY (IC_{50} = 8.5 μM) is degraded during GI digest to RY (IC_{50} = 10.5 μM) and the more potent dipeptide IY (IC_{50} = 2.4 μM) (Saito et al., 1994).

Stability against GI digestion is only the first hurdle that needs to be overcome by bioactive peptides. Once the peptides reach the brush border membrane, they may undergo further cleavage by several membrane anchored epithelial cell intestinal peptidases with varying specificities (Vermeirssen et al.,

Table 3. Contribution of C- and N-terminal amino acids to the gastrointestinal stability of dipeptides (Foltz at al., 2009 with some modifications).

Stability	N-terminal amino acid	C-terminal amino acid
unstable	A, R, C, L, K, M	A, R, C, L, F, W, Y
neutral	Y, F, W	G, H, K, M, D, Q, E, V, I
stable	E, H, I, S, T, V, D, Q	
very stable	N, P, G	N, P, S, T

2004). Aminopeptidase N and A are the major representatives and cleave N-terminal neutral and anionic amino acids, respectively. Additionally, the intestinal brush border contains endopeptidase and dipeptidase activities (Ganapathy and Leibach, 1999; Ganong, 1997). Yet, some amino acids are inhibitors of the brush border membrane peptidases, hence during gastrointestinal digestion inhibitors could be created. In this case the GI halflife of di- and tripeptides might be enhanced which might support absorption (Daniel, 2004).

Low molecular weight substances, such as di – and tripeptides are mainly absorbed via specific transporter proteins (Shimizu, 1999). The most widely studied transporter for small peptides is the peptide transporter 1 (Pept1), a proton-coupled oligopeptide transporter, which is mainly expressed in the apical membrane of small intestinal epithelial cells (Groneberg et al., 2001; Jappar et al., 2010). Pept1 mediates the uptake of di- and tripeptides, but not that of free amino acids or tetrapeptides (Daniel, 2004). As a high-capacity low-affinity influx transporter, Pept1 couples the active uptake of its substrate against the concentration gradient with the downhill uptake of a proton into the enterocyte (Brandsch et al., 2008; Rubio-Aliaga and Daniel, 2008). Pept1 has broad substrate specificity and, hence, also transports structurally similar substances, such as the pharmaceutical ACE-inhibitors captopril and enalapril (Smith et al., 2013). The Caco-2 cell line (human epithelial carcinoma cells) is a frequently applied model of transport via Pept1. Carrier mediated transport using the Caco-2 model was reported for the ACE inhibitory dipeptides AF and IF (Zhu et al., 2008). In a comprehensive study Foltz et al. studied the transepithelial transport of several ACE inhibitory peptides with special reference to IPP and VPP (Foltz et al., 2008). In addition to Caco-2 monolayers these authors also studied jejunal segments in an Ussing chamber for the transport of IPP and VPP. They found a higher transport in the GI model as compared to the cell culture model. Based on these results the authors suggested a paracellular transport of IPP and VPP in parallel to the transport via Pept1 (Foltz et al., 2008). Sun et al., (2009) examined the transport of the antihypertensive peptide KVLPVP by Caco-2 cells. Of note, this transport was not inhibited by the Pept1 substrates Gly-Pro or arphanine A. These data suggest that GI epithelial transport may occur aside from Pept1 and potentially paracellular transport may be involved in the GI absorption of ACE inhibiting peptides (Sun et al., 2009). Along this line, studies employing the ACE inhibitory peptides QIGLFT and TNGIIR suggest a paracellular transport, because transport was only little affected by blocking the Pept1 transporter or using a transcytosis inhibitor (Ding et al., 2014). In contrast, transport was increased in presence of cytochalasin D, a tight junction disrupter (Ding et al., 2016). The discussion on the quantitative contribution of Pept1, transcytosis and paracellular transport of bioactive peptides is unsettled to date. However, it seems clear that the effectiveness of transport of the peptides decreases with increasing chain-length of the peptide (Brandsch et al., 2008; Daniel, 2004; Rubio-Aliaga and Daniel, 2008). Peptide absorption may be increased in infants or persons with gastrointestinal disorders. When the gastrointestinal barrier is not yet fully developed or disturbed later in life, intact peptides and proteins may be absorbed to a greater extent as compared to healthy adults (Vermeirssen et al., 2004).

If intact peptides are absorped, they may undergo hydrolysis by serum peptidases. Studies regarding the inhibitory efficacy and biological variability of W-containing dipeptides on human plasma ACE *ex vivo* showed that there is considerable inter-individual variability with respect to the stability of the ACE inhibitor IW, whereas ACE inhibiting peptides WL and EW did not undergo degradation in serum (Khedr et al., 2015). Similar to gastrointestinal enzymes, serum peptidases and ACE itself may produce potent ACE inhibitors in the circulation by degrading less potent peptide inhibitors of ACE (Vermeirssen et al., 2004; Fujita et al., 2000). Beside the stability of peptides against plasma enzymes, also the elimination via kidney or liver determines plasma half-life. *In vivo* human studies with lactotripeptide-enriched milk determined a plasma half-life of IPP with 26.4 ± 15.1 min (range from 5.7–44.1 min), which is moderately enhanced if volunteers are fed with a standardized breakfast before intake of peptides to 38.6 ± 13.5 min (range from 28.7–58.8 min) (Foltz et al., 2007). Human studies with the ACE inhibitory dipeptides IW and WL reported similar half-lifes. Half-life of WL in human plasma was 39 ± 5 min and that of IW was calculated to 54 ± 12 min (Kaiser et al., 2016). In contrast Matsui et al. showed in their study with SHR a plasma half-life of 2.7 h after intake of VY (Matsui et al., 2004). These authors also measured concentrations of VY in tissue, such as liver, kidney, lung and abdominal aorta. Of note, tissue concentrations were enhanced above baseline levels for more than 9 hours. In addition the mean residence time (MRT), which represents the average time of molecule retention in the body, was calculated. For tissues the residence times of VY were almost twice that in plasma (Matsui et al., 2004). Plasma half-life was also determined for HLPLP and the tripeptides IPP, LPP and VPP. Half-life of these peptides was shorter with less than 12 min for HLPLP and 5–15 min for the tripeptides, respectively (Sanchez-Rivera et al., 2014; van der Pijl et al., 2008).

Plasma concentrations of ACE inhibitory peptides after oral application are typically very low (Kaiser et al., 2016; Matsui et al., 2004; Sanchez-Rivera et al., 2014; van der Pijl et al., 2008). This may be due to poor absorption, rapid degradation by serum peptidases or fast elimination, e.g. by kidney excretion or tissue extraction, respectively. Generally, the concentration of the peptides determined in plasma after oral ingestion is found in the nanomolar range (Kaiser et al., 2016; Matsui et al., 2004; Sanchez-Rivera et al., 2014; van der Pijl et al., 2008). The ACE inhibitory peptide VY was determined in plasma with 3.7 pmol/ml and in tissue with 47.7 pmol/g, given an oral dose of 36 μmol per kg body weight (equal to 10 mg/kg) (Matsui et al., 2004). A nearly twofold amount of the peptide HLPLP (70 μmol equal to 40 mg/kg body weight) was administered orally to rats and the maximum concentration in plasma was about 10 times higher with 35 pmol/ml. The relatively higher bioavailability may be brought about by a higher stability towards proteases (Sanchez-Rivera et al., 2014). However, also in this case absolute plasma concentrations remain low. To avoid the GI passage Pijl et al. (2008) quantified concentrations of the tripeptides IPP, LPP and VPP in plasma after intravenous application and compared it to oral

supplementation. 12 μmol of IPP and VPP, respectively, or 13 μmol LPP were used for both applications. After oral intake 12, 9 and 11 nmol/l (IPP, VPP and LPP) were detected. The concentrations after i.v. application were higher with 126, 107 and 143 μmol/l (IPP, VPP and LPP), respectively, and thus exceeded IC_{50}-values (IPP: 5 μmol/l, VPP: 9 μmol/l, LPP: 9.6 μmol/l). This suggests an effective ACE inhibition *in vivo*, based on *in vitro* IC_{50} estimates, only if the peptides are applied intravenously. In a further study plasma concentrations of the ACE inhibitory peptide HLPLP were quantified after oral intake and i.v. application in Wistar rats (Sanchez-Rivera et al., 2014). Maximum concentration of 331 pmol/ml after i.v. injection of 7 μmol HLPLP and only 35 pmol/ml after oral intake of 70 μmol HLPLP, respectively, were determined. Unfortunately in these studies the effect on SBP and ACE activity *in vivo* was not addressed. Another study reported the IC_{50} of HLPLP *in vitro* with 21.6 mmol/l and an antihypertensive effect (SBP −23.5 mm Hg) after oral intake of 12 μmol (Miguel et al., 2010). Taking the above reported bioavailability (Sanchez-Rivera et al., 2014) of HLPLP and the amount of peptide applied *in vivo* into account this result is unexpected.

Low peptide levels were also measured in human volunteers after oral intake of IW (157 μmol equal to 50 mg) and WL (314 μmol equal to 100 mg), respectively. Maximum plasma concentrations of IW and WL were about 2.4 pmol/ml for IW and 36 pmol/ml for WL, respectively (Kaiser et al., 2016). A dose response study with normotensive volunteers showed increasing VY plasma concentrations of 0.9-1.9 nmol/ml 2 hours after oral intake of 11 (3 mg) and 44 μmol (12 mg) VY, respectively (Matsui et al., 2002b).

To increase the efficacy, peptides could be chemically modified in order to reduce the rate of enzymatic degradation and to increase bioavailability. Modifications such as end changes, glycosylation, alkylation, and conformational changes may therefore have potential for ACE inhibitory peptides (Adessi and Soto, 2002). Using nanoparticles and liposomes for encapsulation will also possibly enhance bioavailability for ACE inhibitory peptides. Furthermore, there is potential for bioactive peptides to be produced by microorganisms through genetic engineering to be delivered to target organs in situ.

To sum up, all studies reported to date determined a low bioavailability after oral administration of ACE inhibitory peptides. Typically only 0.0027% and 0.022% were calculated after oral intake of 50 mg IW and 100 mg WL, respectively (Kaiser et al., 2016). Also the tripeptides IPP, LPP and VPP showed low bioavailability in the range of 0.1% when applied orally to pigs (van der Pijl et al., 2008). The poor bioavailability after oral uptake documented to date raises doubts, whether an antihypertensive effect of ACE inhibitory peptides is realistic. Thus, the next two paragraphs will summarize the evidences supporting a potential biological effectiveness of ACE inhibiting peptides *in vivo*.

4. ACE inhibitory and antihypertensive effects of peptides in animal studies

The efficacy of natural peptides to inhibit ACE activity *in vivo* and lower arterial blood pressure has been addressed in a number of experimental studies (Abubakar et al., 1998; Astawan et al. 1995; Garcia-Tejedor et al., 2014; Groneberg et al., 2001; Li et al., 2007; Martin et al., 2015; Matsufuji et al., 1995; Muruyama et al., 1987; Nakamura et al., 1995; Ruiz-Giménez et al., 2012; Saito et al., 1994; Sato et al., 2002). A frequently used animal model for essential hypertension is the spontaneous hypertensive rat (SHR). The SHR model shows an age dependent development of elevated arterial blood pressure (Pinto et al., 1998) and profound cardiovascular remodeling (Conrad et al., 1995).

Early studies addressing ACE inhibitory peptides focused on IYPRY (*in vitro* $IC_{50} = 8.5$ μM) and YGGY (*in vitro* $IC_{50} = 3.4$ μM), encrypted in sake protein (Saito et al., 1994). There was a significant decrease in blood pressure after a high single oral intake of 100 mg (141 μmol) IYPRY/kg body weight by 15 mm Hg at 4 hours after peptide application. While a maximum decrease of SBP of 21 mm Hg was noted after 24 hours, 72 hours after peptide application baseline blood pressure levels were reached again. Blood pressure in an untreated control group did not change. Unfortunately, the study did not include a placebo control. The dipeptides RY and IY, fragments of IYPRY, also showed antihypertensive effects in SHR (table 4; Saito et al., 1994). As reported above (chapter 3) IYPRY is unstable during simulated gastrointestinal digestion. Hence, it is unclear to which extent the effect on blood pressure is caused by the parent peptide or the daughter peptides, respectively (Saito et al., 1994). No effect on systolic blood pressure (SBP) was determined after oral application of YGGY in SHR (Saito et al., 1994). Because this peptide is an effective ACE inhibitor *in vitro* with an IC_{50} around 16.2 μM (Saito et al., 1994), the missing antihypertensive effect is probably best explained by the instability of YGGY against gastrointestinal digestion (Saito et al., 1994). IPP and VPP have also been assessed for their antihypertensive effect in SHR. After intake of milk fermented with *lactobacillus helveticus* and *saccharomyces cerevisiae*, which contains the tripeptides IPP and VPP, a decrease of SBP by 28 mm Hg (IPP; dosage: 0.3 mg/kg) and 32 mm Hg (VPP; dosage: 0.6 mg/kg) 6 to 8 hours after application was reported (Nakamura et al., 1995). Thereby, the blood pressure lowering effect remained stable for more than 10 hours. Baseline blood pressures were reached again after 24 h. Interestingly, in normotensive Wistar-Kyoto rats (WKY) neither the fermented sour milk nor VPP and IPP had any effect on SBP. No effect on SBP was evidenced even for doses up 10 mg VPP or IPP/kg body weight, which is 17–33 times the dose of these peptides used in SHR (Nakamura et al., 1995). Consecutive studies have addressed the application of sour milk products enchriched with IPP and VPP (Chen et al., 2007; Kim et al., 2010; Michaud et al., 1997; Muguerza et al., 2006; Nakamura et al., 1996; Quiros et al., 2007; Rao et al., 2012; Rodríguez-Figueroa et al., 2013; Rodríguez-Figueroa, 2012; Sipola et al., 2002; Sipola et al., 2001). It has to be realized that such protein hydrolysates may contain other bioactive compounds aside from the target peptide(s). Hence a unique attribution of biological effects caused by the target peptide is difficult for protein hydrolysates. Miguel et al. used egg-white hydrolysate and demonstrated an antihypertensive effect after short-term intake in SHR (Miguel et al., 2005). In agreement with studies on VPP and IPP the same treatment did not affect blood pressure in WKY (Miguel et al.,

Table 4. ACE inhibitory peptides with antihypertensive effect *in vivo* in spontaneously hypertensive rats (SHR).

Peptide	IC_{50} [μM]	Dose [mg/kg]	Max. decrease in SBP [mm Hg]	Reference
VY	26	20 i.v	−7.2*	Matsufuji et al. 1995
		50 i.v	−18*	
IW	0.7	19 oral	−7*	Martin et al. 2015
			−42**	
LW	6.8	10 i.v/60 oral	−45*/ −22*	Fujita et al., 2000
IKP	1.6		−70*/−20*	
IKW	0.21		−50*/−17*	
FKGRYYP	0.55		0*/0*	
AW	15.4	1 oral	−4.8*	Sato et al., 2002
FY	42.3	0,1 oral	−26*	
FP	315	8 oral	−27**	Abubakar et al., 1998
IPA	141		−31**	
GKP	352		−26**	
VYP	288		−24**	
IPP	5	0.3 oral	−28.3*	Nakamura et al., 1995
VPP	9	0.6 oral	−32.1*	
TQVY	18.2	30 oral	−40*	Li et al., 2007
LIWKL	0.47	10 oral	−25.3*	Ruiz-Giménez et al., 2012
VELTP	5.22	10 oral	−20**	Balti et al., 2015
YP		2.0 oral	−32.1*	Maeno et al., 1996
IYPRY	8.5	100 oral	−21*	Saito et al., 1994
IY	2.4	100 oral	−18*	
RY	10.5	100 oral	−17*	

IC_{50} = inhibitor concentration, which is necessary to inhibit 50% of enzyme activity; SBP = systolic blood pressure;*vs. baseline SBP; **vs. control group.

2005). A long-term antihypertensive effect was seen when this hydrolysate was supplemented in SHR for 14 weeks, starting at 6 weeks of age. Blood pressure partially returned toward elevated baseline values when the antihypertensive treatment was discontinued (Miguel et al., 2006). However, SBP still remained lower in animals upon discontinuation of the supplementation if they had received the hydrolysate compared to the control group (Miguel et al., 2006). Jäkälä et al. studied the effect of IPP and VPP in Goto–Kakizaki rats (GKR) (Jäkälä et al., 2009a). This generally normotensive rat model develops hypertension after salt loading and has a metabolic type II diabetic phenotype (Cheng et al., 2001). The development of high blood pressure was attenuated in GKR that received a high salt diet, if a milk product enriched with the tripeptides IPP and VPP was applied for 8 weeks (− 12 mm Hg vs. the control group which received high salt diet and milk without IPP and VPP) (Jäkälä et al., 2009a). Garcia-Tejedor et al., (2015) assessed the antihypertensive effect of the peptides RPYL and DPYKLRP using Wistar rats with induced hypertension by subcutaneous infusion of AngI and AngII, respectively. The oral intake of both peptides (each 10 mg/kg) led to a decrease of blood pressure in the AngI induced hypertension model, while in AngII-stimulated hypertensive rats no effect was observed (Garcia-Tejedor et al., 2015). Matsui et al. addressed the antihypertensive effects of VY in SHR (Matsui et al., 2002a; Matsui et al., 2002b). After oral administration of 10 mg/kg body weight a prolonged reduction of SBP (− 43 mm Hg) for 9 h was determined. A recent study addressed the effects of IW (19 mg/kg body weight) and a whey hydrolysate containing IW (770 mg/kg body weight, which corresponded to 3 mg IW/kg body weight) on arterial blood pressure of SHR that were treated daily via food pellets over 14 weeks (Martin et al., 2015). IW as well as the hydrolysate effectively lowered the age dependent rise of SBP assessed versus a control group of SHR (receiving a standard diet) by 42 ± 3 and 38 ± 5 mm Hg, respectively. The SBP lowering effect was 30 and 26 mm Hg on average for IW and hydrolysate, respectively, versus feeding a nonhydrolysed whey protein (Martin et al., 2015).

The effect of peptides on blood pressure is thought to be mediated via a decrease of peripheral resistance. Few studies have addressed the effects of peptides on other functional cardiovascular parameters. An enhanced endothelium-dependent relaxation of mesenteric arteries and a slight decrease of ACE protein were shown after 8 weeks intake of IPP and VPP during high salt diet (Jäkälä et al., 2009a). Another study found that IPP and VPP preserved endothelium-dependent vessel relaxation in mesenteric arteries isolated from SHR (Jäkälä et al., 2009b). 14 weeks of IW application was shown to attenuate the age-dependent heart mass increase and coronary flow reserve impairment in the SHR model (Martin et al., 2015). Furthermore, isolated aorta preparations from these SHR showed a better endothelium-dependent vessel relaxation and blunted AngI-induced vasoconstriction while IW had no direct effect on vessel tone (Khedr et al., 2017). Thus, there is evidence that application of peptides is associated with a decrease of arterial pressure, preserved endothelial dilator function and a reduction of blood pressure related cardiac hypertrophy.

Experiments under *in vitro* conditions as summarized in Table 2 show ACE inhibitory effects caused by the applied peptides. This generates the rationale for *in vivo* experiments that the change of blood pressure and other cardiovascular parameters is due to the ACE inhibition. However, as multiple effects may induce blood pressure changes, it is important to assess experimentally whether peptide application provokes an inhibition of plasma ACE activity. Although this does not proof a direct cause-effect relationship, this would be regarded as a minimum requirement for potential causality. In contrast to a multitude of studies that have addressed the effects of peptide application on arterial blood pressure, a relatively small number of studies has quantified ACE inhibitory effects of peptides *in vivo*. A recent study reported the effects of IW on ACE activity, matrix-metalloproteinase-2 (MMP-2) expression and activity and renin expression (Martin et al., 2015). Parameters were measured *ex vivo* after a 14-week intake of IW in SHR. IW blunted plasma and tissue ACE activity and reduced MMP-2 activity, which was explained by lower AngII concentrations (Martin et al., 2015; Kopaliani et al., 2016). In addition, gene expression of renin was moderately increased indicating an active feedback within RAS via ACE inhibition (Martin et al., 2015). Another study addressed the effects of VY on ACE activity and AngII concentrations (Matsui et al., 2004). While plasma ACE activity and plasma AngII concentrations were unchanged 6 hours after VY application, significant decreases were found in kidney, abdominal aorta and lung tissues.

In general the blood pressure effects of peptides are similar to those reported for pharmaceutical ACE inhibitors (Balti et al., 2015; Kim et al., 2010; Martin et al., 2015; Miguel et al., 2005; Miguel et al., 2006; Muguerza et al., 2006; Ruiz-Giménez et al., 2012; Sato et al., 2002). Direct comparisons are hampered because different dosages have been used. In a study from Kim et al., (2010) conducted in SHR the antihypertensive effect of captopril was stronger than that of fermented milk containing VPP and IPP. However, the dosage of captopril was 5 times

higher than that of given peptides. Nonetheless, the ACE inhibiting effects in serum and aorta as well as increased plasma NO concentrations and reduction of aortic thickness were similar between IPP and VPP enriched milk and captopril, respectively (Kim et al., 2010). For higher pharmaceutical ACE inhibitor dosages used it is conceivable that enzyme inhibition may be saturated and that a similar effect may be expected for considerably lower dosages. Therefore, it appears essential for future studies to adjust the dosage of peptide ACE inhibitors to those used for pharmaceutical inhibitors. In this regard one study is highlighted which analysed the antihypertensive action of peptides from wakame in SHR versus that of the ACE inhibitor captopril (Sato et al., 2002). The dose of 1 mg/kg of either compound lowered SBP after 6 hours by 18 mm Hg (captopril), 12 mm Hg (IY) and 10 mm Hg (FY). After 9 hours the blood pressure decrease was 14 mm Hg (captopril), 21 mm Hg (IY) and 15 mm Hg (FY), respectively (Sato et al., 2002). Thus, these peptides showed a similar effect as captopril, although the IC_{50} of captopril *in vitro* is in the low nanomolar range, while the IC_{50} values of IY are 5.3 μM and of FY 3.74 μM, respectively (Table 2). This suggests that there are other mechanisms beside ACE inhibition responsible for the measured blood pressure decrease in response to *in vivo* peptide application. The assumption of a cause-effect relationship of bioactive peptides on ACE activity requires biochemically that the putative inhibitor reaches the site of action at a sufficiently high concentration. Data from *in vitro* ACE activity measurements show that peptide concentrations in the micromolar range appear necessary to predict effective ACE inhibition. Plasma concentrations and, in a few cases, tissue concentrations have been assessed in reponse to oral peptide applications. The general result is that most studies were able to demonstrate an increase of the plasma or tissue concentrations. However, plasma concentrations were typically too low to generate effective ACE inhibition *in vivo*. Oral application of 10 mg (36 μmol) VY in a blood pressure lowering dose showed the following concentration changes in plasma and tissue. Plasma levels increased after intake with a maximum after 1 hour (Matsui et al., 2004). In contrast to blood pressure, which was still reduced after 9 hours, plasma concentrations returned to control values. However, concentrations of VY in liver, kidney, lung and aorta were still increased at this time. Compared to VY concentrations in plasma (3.7 pmol/ml) tissue concentration was higher (19.6 – 47.7 pmol/g-tissue) after supplementation (Matsui et al., 2004). Based on these results authors concluded that circulating ACE, which represents less than 2% of whole body ACE (Levitt and Schoemaker, 2006), may not represent the main target for VY. Rather effects of the dipeptide might be due to inhibitory actions on tissue ACE (Matsui et al., 2004). Although the pattern of tissue distribution supports this notion, it has to be pointed out that concentrations of VY determined in plasma and tissue were in the low – middle nanomolar range (plasma: 3.7 pmol/ml or 19.6 – 47.7 pmol/g-tissue). Given the fact that 36 μmol VY per kg body weight had been administered the absorption seems to be too low to yield *in vivo* plasma and tissue concentration above the IC_{50} of the peptide determined for an efficient *in vitro* ACE inhibition (IC_{50} of 26 μmol/l; Matsufuji et al., 1995). As noted above, a blood pressure lowering effect in SHR was shown after intake of the ACE inhibitory tripeptides IPP and VPP (IC_{50} = 5 μM and 9 μM, respectively; Nakamura et al., 1995; Jäkälä et al., 2009a). However, after oral supplementation of these peptides (each 4 mg equal to 12 μmol/kg body weight) plasma concentrations (12 nmol IPP/l and 11 nmol VPP/l) were far below the concentration range that may cause effective ACE inhibition (Table 2). It shall also be pointed out that these concentrations were maximum plasma concentrations, which decayed with half-lifes of 3–5 min (van der Pijl et al., 2008).

In summary, blood pressure effects of peptides have been observed in the majority of experimental studies using rat hypertension models. Up to now, no changes in blood pressure have been reported in response to peptide applications in normotensive animal models (Masuda et al., 1996; Miguel and Aleixandre, 2006; Miguel et al., 2005; Muguerza et al., 2006; Nakamura et al., 1995; Nurminen et al., 2000; Wu and Ding, 2001). In addition to lowering of arterial blood pressure of SHRs, independent measurements of vessel function and heart and vessel remodeling indicate that peptide application may result in structural and functional changes similar to those of pharmaceutical ACE inhibitors. However, the mode of action of theses peptides remains a main unresolved issue: Peptide concentrations determined *in vivo* are too low to explain the observed effects by a direct cause-effect relationship on plasma or tissue ACE in the light of IC_{50} values measured *in vitro* on the isolated enzyme.

5. ACE inhibitory and antihypertensive effects of peptides in human studies

Several clinical trials have been carried out, which addressed the antihypertensive effects of food-derived peptides with ACE inhibiting potential (Aihara et al., 2005; Boelsma and Kloek 2010; Cadée et al., 2007; Fujita et al., 2001; Hata et al. 1996; Hirata et al., 2002; Inoue et al., 2003; Itakura et al., 2001; Jauhiainen et al., 2005; Kajimoto et al., 2002; Kajimoto et al., 2001; Kawasaki et al., 2000; Mizushima et al., 2004; Pins and Keenan, 2003; Nakamura et al., 2004; Seppo et al., 2003; Seppo et al., 2002; Usinger et al., 2012; Xu et al., 2008). Most trials have used fermented milk products containing different concentrations of IPP and VPP. Although the study design varies regarding randomization, blinded approach, placebo-control, study period and baseline treatment with other antihypertensive substances, the majority of studies has reported a significant decrease in blood pressure in the treatment group (Boelsma and Kloek 2010; Hata et al., 1996; Hirata et al., 2002; Jauhiainen et al., 2005; Kajimoto et al., 2002; Mizushima et al., 2004; Nakamura et al., 2004; Seppo et al., 2003). For a consistent assessment of the effect of these products it is necessary to compare blood pressure changes versus a placebo group. It is not sufficient to relate it to the baseline blood pressure levels determined at the start of the trial, because blood pressure has been shown to decrease over study time even in placebo treated groups (Seppo et al., 2003). An early human study done by Hata et al., (1996) studied hypertensive patients taking daily 95 ml fermented milk with 1.1 mg IPP and 1.5 mg VPP. After 4 weeks a significant decrease in SBP of 9.4 mm Hg from baseline value was observed, which was decreased further to 14.1 mm Hg after 8 weeks. Diastolic blood pressure (DBP) was

significantly decreased only after 8 weeks (6.9 mm Hg) (Hata et al., 1996). It should be noted that in this study the overwhelming majority (32 of 36 volunteers) was treated with antihypertensive drugs, which were maintained throughout the trial. Another study tested hypertensive subjects that received fermented milk containing 0.79 mg IPP and 1.12 mg VPP twice daily. After 8 weeks of treatment the study determined a significant decrease in SBP (13.9 mm Hg) and DBP (9.1 mm Hg) compared to placebo (Kajimoto et al., 2002). Some studies record a reduction in blood pressure during the first 2 weeks of peptide intake, which continued to further decrease to a moderate extent (Aihara et al., 2005; Hirata et al., 2002; Kajimoto et al., 2002). Along this line, Aihara et al., (2005) reported that a fermented milk powder with IPP and VPP applied daily in a dose of 4.7 mg IPP and 8.3 mg VPP induced a decrease in SBP of 7.8 mm Hg after the first week. Blood pressure had decreased by 10.5 mm Hg after 2 weeks and by 11.2 mm Hg after 4 weeks of trial (Aihara et al., 2005). Surprisingly, the antihypertensive effect was present even 4 weeks after discontinuation of intake of milk powder preparation. Such an extended effect of pressure lowering has not been reported in other studies addressing the effects of bioactive peptides. Furthermore, the result differs from those reported for several antihypertensive drug treatments, which are known to result in an early elevation of blood pressure after discontinuation of treatment (Hirata et al., 2002; Kajimoto et al., 2002; Kajimoto et al., 2001; Nakamura et al., 2004; Seppo et al., 2002).

Given the studies reported above, it appears that VPP and IPP are effective in reducing blood pressure in humans, who exhibit at least a mildly elevated blood pressure. In none of the clinical trials, in which normotensive controls have been included, a significant blood pressure change has been reported (Itakura et al., 2001; Kajimoto et al., 2001). Even a daily dose of 29.2 mg of VPP and IPP for 7 days had no effect on blood pressure in normotensives (Yasuda et al., 2001). This is in line with results obtained in normotensive animals (see chapter 4) and it is in contrast to human clinical studies using pharmaceutical ACE inhibitors, which induce a mild decrease of blood pressure even in normotensive subjects. The blood pressure lowering effect of pharmaceutical ACE inhibitors is augmented in subjects with elevated blood pressure (Law et al., 2003). The augmented effectiveness of bioactive peptides in hypertensive individuals is most comprehensively documented for VPP and IPP (Aihara et al., 2005; Cicero et al., 2013; Cicero et al., 2011; Hata et al., 1996; Hirata et al., 2002; Hirota et al., 2007; Itakura et al., 2001; Jauhiainen et al., 2010; Jauhiainen et al., 2005; Kajimoto et al., 2001; Mizushima et al., 2004; Nakamura et al., 2009; Nakamura et al., 2004; Seppo et al., 2004; Seppo et al., 2002; Yasuda et al., 2001). Similar antihypertensive effects have been reported for a few other peptides or protein hydrolysates. In the case of peptide applications, e.g. VY and LKPNM, the compound identities are given. However, when using hydrolysates the peptide identity is not always resolved (Cadée et al., 2007; Fujita et al., 2001; Inoue et al., 2003; Kawasaki et al., 2000; Pins and Keenan, 2003). Recently, the original studies habe been evaluated in two meta-analyses (Pripp, 2008; Wang et al., 2010). The consensus is that food-derived antihypertensive peptides cause a significant, although low, decrease in blood pressure. This effect on blood pressure appears greater in hypertensive subjects than in prehypertensive ones (Pripp, 2008; Wang et al., 2010). However, it must be acknowledged that there are studies, which did not find an effect of VPP and IPP on blood pressure (Engberink et al., 2008; Wuerzner et al., 2009). Engberink et al., (2008) showed in a comprehensive study including 135 volunteers with elevated blood pressure no difference in blood pressure compared to placebo after intake of VPP and IPP for 8 weeks. In addition to stable blood pressure values, plasma ACE activity and plasma AngII concentrations were unchanged (Engberink et al., 2008). Matsufuji et al., (1995) analysed the antihypertensive effect of the dipeptide VY in mildly hypertensive volunteers. A decrease of arterial blood pressure was not observed, although an effective blood pressure reduction has been reported in the SHR model (Matsufuji et al., 1995; Matsui et al., 2002a). Data reported for VY seem inconclusive, because a significant decrease of blood pressure following application of VY in mild hypertensive subjects has been reported in another study (Kawasaki et al., 2000).

As it has been pointed out for animal experiments (chapter 4), proving a cause-effect relationship requires that a quantitatively sufficient plasma peptide concentration is reached after oral application. A few studies have analysed pharmacokinetic parameters partially along with blood pressure changes (Foltz et al., 2007; Wuerzner et al., 2009). In a small study on 12 healthy normotensive volunteers the effects of daily application (over 7 days) of a fermented milk preparation containing 4.5 mg (13.8 μmol) IPP and 6.6 mg (21.2 μmol) VPP for 7 days was studied (Wuerzner et al., 2009). Concentrations of the peptides were measured after the first intake and at the end of the study period. Quantification of plasma concentrations was only possible for IPP, because VPP concentrations were below quantification limit. Plasma levels of IPP increased after the first intake and reached a maximum in the low nanomolar range after 30 minutes. Plasma levels returned to baseline levels during the following 2 hours. No increase of the peptide plasma concentration was seen after 7 days of daily intake. Furthermore, no effect was seen on plasma ACE activity either directly after the intake or up to 8 hours (Wuerzner et al., 2009). This would be an expected result given the nanomolar plasma concentration. Similar low plasma concentrations of IPP reported Foltz et al., (2007) in 6 healthy subjects after intake of a beverage containing IPP, VPP and LPP (Foltz et al., 2007). Oral ingestion of the dipeptide VY resulted in a small dose-dependent rise of plasma concentration with a maximum at 1.25 nmol/l for a dose of 6 mg (21.4 μmol) VY. Maximum plasma concentration was 2.04 nmol/l when a dose of 12 mg (42.8 μmol) VY was given (Matsui et al., 2002a). Measurements in healthy normotensive volunteers showed the intact absorption of the ACE inhibitory peptides IW and WL after a single intake of 50 mg (157 μmol) IW or 100 mg (315 μmol) WL, respectively (Kaiser et al., 2016). Plasma levels of these dipeptides reached nanomolar concentrations. In parallel with this small, although significant, rise of peptide plasma concentrations plasma ACE activity decreased by 32% and 22% after IW and WL administration, respectively. Administration of the corresponding single amino acids I and W had no effect on plasma ACE activity and peptide

concentrations. Two hours after IW and WL application baseline plasma concentrations were reached again. However, a small ACE inhibiting effect remained present up to 3 h post application (Kaiser et al., 2016). In addition to arterial blood pressure a few clinical studies have determined other hemodynamic parameters (Cicero et al., 2011; Hirota et al., 2007; Jauhiainen et al., 2010; Nakamura et al., 2009). In 52 human volunteers with high-normal blood pressure or first-degree hypertension the effects of a daily intake of a juice enriched with IPP and VPP (total 3 mg) for 6 weeks was studied on blood pressure, pulse wave velocity, stroke volume, stroke volume index, acceleration index and velocity index (Cicero et al., 2011). In addition to a reduction of blood pressure (SBP −4.8 mm Hg vs. placebo; DBP no significant change) a significant improvement in pulse wave velocity (− 0.66 m/s) was reported. Intake of IPP and VPP, but not placebo, was associated with a mild but significant change in the stroke volume and stroke volume index, the acceleration index (ACI) and velocity index (Cicero et al., 2011).

Decreased arterial stiffness, measured by the augmentation index, was shown after administration of a drink and tablets containing VPP and IPP, respectively (Jauhiainen et al., 2010; Nakamura et al., 2009). The authors reported that the decrease of the augmentation index was more pronounced in males than in females (Jauhiainen et al., 2010). Another study analyzed the effects of VPP and IPP (3.87 mg of IPP and 3.42 mg of VPP per day) given for one week on the reactive hyperemia of the forearm measured by plethysmography in mild hypertensive patients. While the reactive hyperemia was significantly improved, arterial blood pressure remained unchanged. The study was interpreted to indicate that vascular endothelial function was enhanced. Because blood pressure was unchanged, it was further concluded that the improvement of the vascular endothelial function following VPP and IPP application occurred independent of hemodynamic changes (Hirota et al., 2007).

In summary, evidence obtained in clinical studies is largely compatible with results from animal experiments. While blood pressure lowering effects have been documented for peptide applications in hypertensive subjects, no blood pressure changes have been detected in normotensive volunteers. Bioactivity studies detected increases in plasma peptide concentrations after oral ingestion. The time course of elevated plasma concentrations goes along with a decrease of plasma ACE activity. However, it needs to be pointed out that the rise of plasma peptide concentrations seems to be too small and quantitatively not suffient to explain the decrease of plasma ACE activity assuming that IC_{50}-values determined for ACE *in vitro* are also relevant for *in vivo* conditions.

6. The current dilemma and future directions

As evident from the preceding paragraphs a large number of food-derived peptides with ACE inhibitory effect *in vitro* have been studied over the last decade. Using several of these peptides antihypertensive effects have been documented in experimental animal models and in human trials. Beside the fact that not all *in vivo* studies support the antihypertensive effect (Engberink et al., 2008; Wuerzner et al., 2009), a general shortcoming in the current mechanistic concept is the discrepancy between *in vitro* IC_{50} values, *in vivo* plasma or tissue concentrations of peptides and the observed effects on arterial blood pressure. Currently, this discrepancy precludes to accept a clear cause and effect model. A major uncertainty seems to be brought about by the still unclear extent of GI resorption. To date only a few studies have investigated the effects of ACE inhibitory peptides after i.v. application. Unfortunately, these studies lack measurements of plasma concentrations of peptides or plasma ACE activity. Only two studies (van der Pijl et al., 2008; Sanchez-Rivera et al., 2014) have measured plasma concentrations of the peptides after i.v. and oral application. However, these studies did not investigate blood pressure or ACE inhibition. It is not surprising that peptide concentrations were higher after i.v. application. Yet only in one study (van der Pijl et al., 2008) *in vivo* concentrations were reached above the *in vitro* IC_{50}-estimate of the peptides under study. The currently reported plasma concentrations are believed to represent free concentrations but the extent of likely protein binding is unknown. More detailed information on pharmacokinetics including elimination, plasma and tissue half-life are urgently needed to improve understanding of the precise mode of action. Because of the existing gap between documented plasma concentrations of peptides, *in vitro* IC_{50}-values of ACE inhibition and observed blood pressure effects also alternative explanations should be investigated. Alternative modes of action of food-derived peptides reported in literature include interactions with bradykinin receptors (Barba de la Rosa et al., 2010; Benzing et al., 1999; Deddish et al., 2002; Fujita et al., 1995; Marcic and Erdös, 2000; Marcic et al., 1999; Minshall et al., 1997; Scruggs et al., 2004), Ca^{2+} channels (Tanaka et al., 2008; Wang et al., 2010), opioid receptors (Meisel and FitzGerald, 2000; Jauhiainen and Korpela, 2007; Nurminen et al., 2000; Hernńdez-Ledesma et al., 2011), inhibition of renin activity (Fernandez-Musoles et al., 2013; Girgih et al., 2014; He et al., 2014; Li and Aluko, 2010; Onuh et al., 2015; Udenigwe et al., 2009), endothelin-converting enzyme (Kedzierske and Yanagisawa, 2001; Maes et al., 2010) and effects on sympathetic nerve activity (Usinger et al., 2010).

Bradykinin, which activates endothelial nitric oxide synthase (eNOS), is augmented by ACE inhibition. A cross-talk between ACE-inhibitor complex and bradykinin B2 receptors has been described, although the mechanisms behind this interaction are still unclear (Benzing et al., 1999; Deddish et al., 2002; Marcic and Erdös, 2000; Marcic et al., 1999; Minshall et al., 1997). So far only a few studies have analysed the direct interaction of ACE inhibitory peptides and bradykinin receptors (Barba de la Rosa, 2010; Fujita et al., 1995; Scruggs et al., 2004). A peptide, which mediates a hypotensive effect through interaction via bradykinin B2 receptors is ovokinin 2–7 (RADHPF) (Scruggs et al., 2004). Peptides FRADHPFL and RADHPF induced endothelium-dependent vasorelaxation, which involved bradykin B1 receptor and prostacyclin (Fujita et al., 1995). Amaranth trypsin-digested glutelins induced NO production in coronary endothelial cells and NO-induced vasodilatation in isolated rat aortic rings by binding to the B2 receptor (Barba de la Rosa et al., 2010).

For some peptides an inhibitory effect on calcium channels has been suggested (Tanaka et al., 2008; Wang et al., 2010). The ACE inhibitory peptides WH, HW, WL and WV induce a vasodilating effect in KCl-contracted aortic rings, which is endothelium-independent (Tanaka et al., 2008). This effect was not caused by ACE inhibition, because co-incubation with a synthetic ACE inhibitor did not affect the vasodilating effect. Rather, the vasodilatatory effect of WH was caused via blocking L-type Ca^{2+} channels as indicated by the effect of WH on intracellular Ca^{2+} level in smooth muscle cells and the interference of the response with Ca^{2+}-channel blockers (Tanaka et al., 2008; Wang et al., 2010).

Opioid like activities of food-derived peptides have been suggested to rely on binding to opioid receptors found in the nervous, endocrine and immune system. Among other regulatory processes opioid receptor modulation may cause effects on blood pressure (Jauhiainen and Korpela, 2007; Meisel and FitzGerald, 2000). Application of the peptide YGLF (which also shows an ACE inhibitory action (Mullally et al., 1996)) caused a blood pressure reduction in SHR and Wistar rats, respectively, which seemed to be mediated via opioid receptors, because the response was antagonized by the specific opioid receptor antagonist naloxone (Nurminen et al., 2000). Opiod receptors are also located in the intestinal tract, which opens up a possibility of interaction between food-derived peptides and these receptors (Hernandez-Ledesma et al., 2011). If such receptor interactions would result in the modulation of sympathetic nerve activity, this might explain antihypertensive effects despite low plasma concentrations of peptides.

Renin is one of the key players in RAS and renin inhibition leads to lower AngI concentrations. Peptides from food protein inhibiting ACE and renin are found in enzymatic flaxseed fractions and pea protein hydrolysates (Girgih et al., 2014; He et al., 2014; Udenigwe et al., 2009). Li and Aluko (2010) showed that IW and LW also moderately inhibit renin activity, whereas VW and AW exerted an action restricted to ACE. The pronounced blood pressure lowering activity of IW (Li and Aluko, 2010; Martin et al., 2015) might result from this dual action. Beside ACE inhibition and RAS modulation, effects of peptides on endothelin converting enzyme (ECE) have been described (Fernandez-Musoles et al., 2013). The β-lactoglobulin-derived peptide ALPMHIR suppressed the release of the vasoconstrictor peptide endothelin-1 (ET-1) in cultured endothelial cells (Maes et al., 2004). ET-1 mediates a strong vasoconstriction via ETa- and ETb-receptors located on smooth muscle cells. Additonally, this peptide may also induce relaxation via direct binding to endothelial ETb-receptors, which stimulate nitric oxide production
(Fernandez-Musoles et al., 2013). The *in vivo* effects of ECE inhibition by food-derived peptides on blood pressure are currently unclear.

The effects of daily intake of fermented milk over 8 weeks on RAS and sympathetic nerve activity were studied in volunteers with borderline hypertension (Usinger et al., 2010). The fermented milk preparation containing IPP and VPP had no significant effects on blood pressure and RAS parameters. However, the plasma noradrenaline response determined during a tilt test was declined, which was suggested to indicate a modulating effect on sympathetic nerve activity (Usinger et al., 2010).

Thus, there is limited evidence that food-derived peptides might cause antihypertensive effects aside from ACE inhibition. These alternative modes of action should be critically evaluated in future studies.

In conclusion, although not all experimental studies and clinical trials support an antihypertensive action of food-derived peptides with *in vitro* ACE inihibiting activity, the majority of studies support this view (Pripp, 2008; Xu et al., 2008). In contrast, the lack of hypotensive effects of these peptides has been documented under conditions of normotension (Itakura et al., 2001; Kajimoto et al., 2001). Thus, if antihypertensive effect of a peptide can be documented with appropriate controls (placebo, time), the effect is highly likely to be restricted to individuals with enhanced arterial blood pressure. Also, side effects commonly seen with pharmaceutical ACE inhibitors have not been observed with food-derived peptides, which could be explained by the low circulating plasma concentration that are unlikely to cause typical side effects. Thus, the application of these peptides appears to be safe. Nonetheless, in consideration of peptide application versus pharmaceutical ACE inhibitor treatment it seems of utmost importance to elucidate in detail the mode of action of food derived peptides mediating the blood pressure blunting effect. The acceptance of this alternative approach as a means to diminish or delay the early onset of hypertension will largely depend on the clarification of the cause-effect relationship. Given the fact that hypertension is the leading risk factor for all cause mortality of noncontagious diseases worldwide (World Health Organization, 2014) the efforts to be undertaken seem worth it to further developing innovative food additives with blood pressure lowering and vessel protective actions.

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