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Ala-Val-Phe and Val-Phe: ACE inhibitory peptides derived from insect protein with antihypertensive activity in spontaneously hypertensive rats

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ABSTRACT

In this study, we evaluated the stability/bioavailability and *in vivo* antihypertensive activity of the tripeptide, Ala-Val-Phe, that was recently purified from insect protein (*Spodoptera littoralis*; Lepidoptera) and that showed *in vitro* angiotensin converting enzyme (ACE) inhibitory activity. This tripeptide is partly hydrolyzed by mucosal peptidases to Val-Phe, a more potent *in vitro* ACE inhibitor. In organ bath experiments using rat aorta, Val-Phe showed ACE inhibition, while Ala-Val-Phe did not. Single oral administration (5 mg/kg body weight) to spontaneously hypertensive rats led to a significant decrease in blood pressure for both peptides. Docking experiments indicated an active character for Val-Phe and an inactive character for Ala-Val-Phe as potential inhibitors of human ACE. From our results, it can be suggested that after oral administration of Ala-Val-Phe, Val-Phe is released by *in vivo* peptidases and is responsible for *in vivo* activity of Ala-Val-Phe. To the best of our knowledge this is the first report of *in vivo* antihypertensive activity of peptides derived from insect protein.

1. Introduction

Hypertension or high blood pressure is a major risk factor for cerebro- and cardiovascular disorders. Therefore, blood pressure should be controlled and properly regulated in hypertensive patients [6]. A moderate control of blood pressure in hypertension can be obtained by a nutritional approach. Functional foods, containing ACE inhibitory peptides, may be part of this approach [11]. Many *in vitro* ACE inhibitory peptides have been reported [14,34]. However, in spite of their *in vitro* activity, some of these peptides fail to show antihypertensive activity after oral administration to spontaneously hypertensive rats (SHR) [8,18]. For example, Phe-Lys-Gly-Arg-Tyr-Tyr-Pro isolated from the thermolysin digest of chicken muscle showed an *in vitro* IC_{50} value of 0.55 μM, but no antihypertensive activity could be observed after oral administration (60 mg/kg BW) to SHR [7]. On the other hand, the opposite has also been observed: peptides showing a low *in vitro* ACE inhibitory activity exerted a high *in vivo* antihypertensive effect [21,34]. Nakashima et al. [21] tested eight oligopeptides derived from porcine skeletal muscle protein. Pro-Pro-Lys demonstrated the highest antihypertensive activity in SHR after oral administration although it showed the lowest *in vitro* ACE inhibitory activity (IC_{50} value > 1000 μM). This important lack of correlation between *in vitro* ACE inhibitory activity and *in vivo* antihypertensive activity might be explained by the bioconversion/availability of ACE inhibitory peptides or by the possible antihypertensive mechanisms other than ACE inhibition which are influenced by these peptides [17].

After oral administration, ACE inhibitory peptides can only exert their biological activity *in vivo* if they reach the bloodstream in active form. The resistance of the peptides against digestive proteases is crucial, as well as the intestinal absorption and transportation in the blood. Vermeirssen et al. [32] reviewed the possible activation and inactivation sites of ACE inhibitory peptides in the human body. In addition, the hydrolysis pattern of the peptides by ACE itself is important. Depending on this interaction, ACE inhibitory peptides can be classified into three categories: inhibitor-type, substrate-type and prodrug-type inhibitors [8]. For the inhibitor-type, no difference in IC_{50} values can be seen with or without preincubation with ACE. A preincubation

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with ACE leading to an increase in IC_{50} value (decrease in activity) indicates a degradation of the peptides by ACE, which is similar to ACE substrates and is therefore called the substrate-type. A prodrug-type inhibitor is hydrolyzed by ACE to release the true inhibitor; hence preincubation with ACE leads to a decrease in IC_{50} value. Both the inhibitor-type and prodrug-type peptides are expected to show long-lasting antihypertensive activity after oral administration to SHR. Hence it should be remarked that antihypertensive effects can only be reliably assessed by *in vivo* experiments using SHR [6]. A second remark is that when an antihypertensive activity of peptides has been proven, the mechanisms underlying this activity have been rarely examined [28]. However, increasing evidence is being provided that mechanisms different from ACE inhibition may be involved in the antihypertensive effect of peptides [15,19,20].

In a previous study, we evaluated the *in vitro* ACE inhibitory activity of the nonwater soluble protein fraction of the insect *Spodoptera littoralis* (Lepidoptera). Various enzymes were used to hydrolyze the protein, and the gastrointestinal digest showed a strong ACE inhibitory activity with an IC_{50} value of 320 μg/ml [30]. This digest was fractionated using several chromatographic steps and the ACE inhibitory tripeptide Ala-Val-Phe was purified [30]. The objectives of this study were to investigate the characteristics of Ala-Val-Phe and of its fragment Val-Phe. The type of inhibitor as categorized by Fujita and Yoshikawa [8] and the resistance to digestive proteases were determined. Organ bath experiments were used to detect ACE inhibitory activity in isolated rat aorta. In addition, after oral administration of Ala-Val-Phe and Val-Phe to SHR, the actual *in vivo* antihypertensive effect was assessed. Docking experiments were performed to offer a molecular basis for the inhibitory activity of the peptides.

2. Materials and methods

2.1. Products

Hippuryl-L-histidyl-L-leucine (HHL), ACE (from rabbit lung), o-phthaldialdehyde (OPA), angiotensin I, angiotensin II, acetylcholine, N-nitro-L-arginine (NNA), pepsin, trypsin, α-chymotrypsin and peptidases from porcine intestinal mucosa were purchased from Sigma–Aldrich (Bornem, Belgium). The synthesized peptides, Ala-Val-Phe and Val-Phe were purchased from Peptisyntha (Brussels, Belgium). Lisinopril was provided by Merck & Co. (Rahway, NJ).

2.2. In vitro ACE inhibitory activity

The determination of *in vitro* ACE inhibitory activity was performed by the spectrophotometric method described by Chang et al. [2] with slight modifications. The method is based on a selective chromogenic reaction for histidyl-leucine using o-phaldialdehyde. For each assay, 100 μl of protein hydrolysate, 100 μl of HHL solution (in 15 mM in 20 mM sodium tetraborate and 0.3 M NaCl, pH 8.3) and 100 μl of ACE (40 mU/ml in 15 mM in 20 mM sodium tetraborate and 0.3 M NaCl, pH 8.3) were incubated for 2 h at 37 °C. The enzymatic reaction was terminated and the chromogenic reaction started by adding 2 ml of alkaline OPA reagent. The OPA reagent consists of 1.65 ml OPA (10 mg/ml in ethanol), 1.65 ml of 2-mercaptoethanol (5 μl/ml in ethanol) and 73 ml of sodium borate buffer (0.1 M sodium tetraborate and 0.2 M NaOH). After an incubation period (20 min, 25 °C), the absorbance was measured at 390 nm. Appropriate blanks were run. The IC_{50} value, defined as the concentration of sample that inhibits 50% of ACE activity, was determined by nonlinear regression analysis of the dose–response curve as described previously [29].

2.3. Stability of Ala-Val-Phe to digestive proteases

To simulate the human gastrointestinal digestion process, a subsequent hydrolysis using pepsin, trypsin and α-chymotrypsin was performed [29]. The digestion in the stomach was simulated by lowering the pH to 2 with HCl (4 M), adding pepsin (E/S, 1/12) and incubating for 2 h at 37 °C. Trypsin and α-chymotrypsin (E/S, 1/12) at pH 6.5 (with NaOH, 10 M) and incubation for 2.5 h at 37 °C, simulated the small intestine phase. Porcine mucosal peptidases contain a general proteolytic and aminopeptidase activity similar to the enterocytes in the human body. Hydrolysis with these mucosal peptidases was done during 2 h at 37 °C and pH 7.4 with an enzyme to substrate ratio of 1/50. After hydrolysis, the pH was adjusted to 4 with HCl.

The *in vitro* ACE inhibitory activity of the reaction mixtures was determined as described above. In addition, to evaluate the effect of hydrolysis and the hydrophobicity of the peptides, the reaction mixtures were applied onto a C18 column (Lichrosorb, 10 μm RP18, 250 mm × 4.6 mm, Varian, Sint-Katelijne-Waver, Belgium). Val-Tyr (10 mg/ml) was added to the reaction mixtures as internal standard. A linear gradient elution using acetonitrile and trifluoroacetic acid (TFA) was used. Eluent A consisted of 0.1% TFA in HPLC grade water; eluent B of 0.07% TFA in HPLC grade acetonitrile. Before injection of 50 μl sample, the column was conditioned with 98% of eluent A. The concentration of eluent B was increased to 20% in 10 min. During the next 25 min, the concentration of eluent B was further increased to 45%. A flow rate of 1 ml/min was applied and UV absorbance was monitored at 214, 220 and 250 nm using a diode array detector (Thermo electron).

2.4. Stability of peptides to ACE

The stability of peptides to ACE was tested by preincubating the peptides with ACE for 1 h at 37 °C before the substrate HHL was added in the *in vitro* ACE inhibitory activity assay. Comparison of the IC_{50} values with and without preincubation with ACE indicates the type of inhibitor. Student's *t*-test was used to indicate significant differences.

2.5. ACE inhibitory and Ang II receptor blocking activity determined in organ baths using rat aortic rings

Thoracic aortic rings from male Wistar rats were prepared and mounted in organ baths as described previously [28]. In order to test their contractile capacity and the functionality of the endothelium, physiological solution in the baths was replaced with KCl-rich solution (KCl 100 mM) and the relaxation caused by acetylcholine (1 μM) in KCl-precontracted rings was evaluated. The effect of Ala-Val-Phe, Val-Phe and lisinopril on the contraction evoked by Ang I or Ang II was tested. Therefore, the aortic rings were incubated for 30 min in the presence or absence of peptide or lisinopril in physiological solution containing 100 μM of the NOS inhibitor N-nitro-L-arginine (NNA). After the incubation period, contraction of the rings was evoked by Ang I or Ang II to reach cumulative concentrations of 1–100 nM [28]. Results are expressed as mean ± SEM, and the number of determinations (n) is given. Agonist potency is expressed as a pD_2 value, which is the negative logarithm of the EC_{50} value (concentration of agonist resulting in 50% of the maximum response). Student's *t*-tests and ANOVA were used to compare data and differences with p-values <0.05 were considered to be significant. Concentration–response curves were analyzed by nonlinear regression (GraphPad Prism v4, GraphPad Software Inc., San Diego, CA).

2.6. Antihypertensive activity after oral administration using spontaneously hypertensive rats

Male spontaneously hypertensive rats (SHR) between 9 and 13 weeks old, weighing 230–290 g, were purchased from Charles River Laboratories (L'Arbresle Cedex, France). The SHR were kept at 24 °C with a 12 h dark–light cycle. They were fed a standard laboratory diet, and tap water was freely available. This study has the ethical approval of the animal experimental committee of the Université Catholique de Louvain (ref. 2003/12/FMD/UCL/001). The ACE-inhibitory peptides were dissolved in 0.5 ml of tap water at a dose of 5 mg/kg body weight (BW), and orally administered by gavage. Control rats were administered the same volume of tap water. Systolic blood pressure (SBP) was measured by the tail-cuff method [16] before (time 0) and 2, 4, 6, 8 and 24 h following oral administration of the sample. Before each series of measurements, SHR were conditioned on a 37 °C hot plate for 10 min to make the pulsations of the tail artery detectable. SBP was measured using a Physiograph desk model with programmed electro-sphygmomanometer and pneumatic pulse transducer (Narco bio-systems, Austin, TX). To guarantee the reliability of the measurements, the rats were accustomed to the procedure a week before starting the experiments. Changes in SBP from zero time were expressed as mean ± SEM. Student's *t*-tests were used to indicate differences against the control.

2.7. Molecular docking of Ala-Val-Phe and Val-Phe in human tACE

Human testicular ACE (tACE) in complex with captopril (RCSB PDB code 1UZF) [22] was used as a template for docking experiments with the stable conformations of Val-Phe and Ala-Val-Phe peptides. Dipeptide Val-Phe and tripeptide Ala-Val-Phe were built using PyMol and their stable conformations were calculated using the steepest descent/conjugate gradient algorithms in the Discover3 forcefield. Docking was performed with InsightII. Clipping planes of tACE were rendered using the UCSF Chimera package [24]. Molecular cartoons were drawn with PyMol (http://pymol.sourceforge.net).

3. Results and discussion

In a recent study, we identified a new *in vitro* ACE inhibitory tripeptide, namely Ala-Val-Phe, from the gastrointestinal digest of the nonwater soluble protein fraction of *S. littoralis* [30]. Before the actual *in vivo* antihypertensive activity was tested, we evaluated the stability of the peptide to digestive proteases and to ACE as these are crucial factors influencing *in vivo* activity.

3.1. Gastrointestinal resistance of Ala-Val-Phe

To evaluate the resistance of Ala-Val-Phe to digestive proteases after oral administration, Ala-Val-Phe was hydrolyzed using a simulated gastrointestinal digestion followed by digestion with mucosal peptidases. The RP-HPLC analysis of each reaction mixture showed that no hydrolysis of Ala-Val-Phe occurred after gastrointestinal digestion using pepsin, trypsin and α-chymotrypsin. This resistance could be expected as Ala-Val-Phe was purified from *S. littoralis* protein after gastrointestinal digestion [30]. In contrast, peptidases from porcine intestinal mucosa hydrolyzed Ala-Val-Phe and released Val-Phe, although a significant amount of Ala-Val-Phe remained after 2 h digestion at 37 °C. The percent of residual Ala-Val-Phe was estimated at 77.3 ± 0.1% by the area under the curve on the HPLC chromatogram. The *in vitro* ACE inhibitory activity of the nonhydrolyzed and gastrointestinal digest of Ala-Val-Phe was identical, while gastrointestinal digestion followed by mucosal digestion led to a slight increase in *in vitro* ACE inhibitory activity compared to control, although this increase was not significant ($p = 0.13$) (Table 1).

Table 1
Resistance of Ala-Val-Phe to digestive proteases.

	IC_{50} (μM)	Log IC_{50} ± SEM
Nonhydrolyzed peptide	660	2.82 ± 0.01
Gastrointestinal digestion	655	2.82 ± 0.01
Gastrointestinal + mucosal digestion	498	2.70 ± 0.05

These results indicate that ingested peptides might be broken down by digestive enzymes *in vivo* which was reported previously [1]. This break down can result in either an increase or decrease in activity. Maeno et al. [18] reported that the heptapeptide, Lys-Val-Leu-Pro-Val-Pro-Gln, purified from casein hydrolysate, showed a rather low *in vitro* ACE inhibitory activity ($IC_{50} > 1000$ μM). However, the shorter peptide Lys-Val-Leu-Pro-Val-Pro has a high *in vitro* ACE inhibitory activity (IC_{50} value = 5 μM) and can be released from the heptapeptide by pancreatic digestion. Therefore, it could be suggested that the antihypertensive activity observed after oral administration of the heptapeptide to SHR might be due to the hexapeptide which is released during gastrointestinal digestion *in vivo*. This agrees with Katayama et al. [13] who evaluated the digestive protease resistance of 9mer, a peptide isolated from porcine skeletal troponin; the 9mer was partly digested by pepsin, trypsin and chymotrypsin.

As Ala-Val-Phe is hydrolyzed to Val-Phe by mucosal peptidases, we included Val-Phe in our further evaluation. Val-Phe has been previously reported by Suetsuna et al. [26]. A hot water extract of wakame (*Undaria pinnatifida*) was dialyzed and fractionated by ion-exchange chromatography. Ten ACE inhibitory peptides were identified, including Val-Phe of which the *in vitro* ACE inhibitory activity was determined with an IC_{50} value of 43.7 μM.

3.2. Stability of Ala-Val-Phe and Val-Phe to ACE

The stability of Ala-Val-Phe and Val-Phe to ACE was evaluated by preincubating the peptides with ACE for 1 h before the substrate HHL was added to the reaction mixture. Lisinopril was also tested for comparative purposes. The IC_{50} values with and without preincubation are given in Table 2. Both peptides, as well as lisinopril, are true-type inhibitors as the IC_{50} values do not significantly change by preincubation with ACE. According to Fujita and Yoshikawa [8], these results suggest that the peptides should exert antihypertensive activity after oral administration. However, *in vivo* activity is also depending on other factors such as resistance of the peptides to digestive proteases and intestinal absorption.

Table 2
Stability of the peptides and lisinopril to ACE.

	−Preincubation		+Preincubation		Type of inhibitor
	IC_{50} (μM)	Log IC_{50} ± SEM	IC_{50} (μM)	Log IC_{50} ± SEM	
Ala-Val-Phe	1430	3.16 ± 0.07	1374	3.14 ± 0.08	True inhibitor
Val-Phe	120	2.08 ± 0.08	144	2.14 ± 0.14	True inhibitor
Lisinopril	0.029	−1.54 ± 0.27	0.017	−1.78 ± 0.18	True inhibitor

3.3. Hydrophobicity of Ala-Val-Phe and Val-Phe

The binding of a peptide to ACE is positively influenced by hydrophobic amino acids forming the peptide, especially the hydrophobicity of the C-terminal amino acids is decisive for the ACE inhibitory activity [10,25]. The hydrophobicity of a peptide can be estimated to a certain extent by the amino acids in the peptide. The amino acid '*z*-scores', obtained by principal component analysis, have been used to develop QSAR models of ACE inhibitory peptides. The *z*1-score is related to hydrophobicity and is −4.92, −2.69 and 0.07 for Phe, Val and Ala, respectively [33]. This indicates that our tri- and dipeptide meet the requirements of hydrophobicity necessary for activity.

In addition, we evaluated the hydrophobicity of the di- and tripeptide by RP-HPLC. The respective retention times of Ala-Val-Phe and Val-Phe were 23.8 and 21 min, indicating that Ala-Val-Phe is slightly more hydrophobic than Val-Phe, due to the extra alanine.

3.4. ACE inhibition and Ang II receptor blocking in isolated rat aorta

By using organ bath experiments, we evaluated the ACE inhibitory activity of Ala-Val-Phe and Val-Phe in isolated rat aorta. Rat aortic rings were contracted with Ang I or Ang II (cumulative concentrations of 1–100 nM) and the effect of a preincubation with the peptides was evaluated.

We started the study by using lisinopril as a reference. Fig. 1A shows the effect of lisinopril on the concentration–response curves to Ang I. A significant shift of the curve is observed for the three lisinopril concentrations used. As indicated in Table 3, the Ang I pD_2 values are significantly different for all concentrations. The maximum response, expressed as % of KCl response, showed no significant difference. This study shows that ACE inhibition can also be determined in isolated rat aorta, which is more closely related to *in vivo* conditions than the *in vitro* experiments. In the organ baths, rat aortic rings are used which are a complex system where multiple interactions can take place. In contrast *in vitro* assays are isolated systems where only the ACE reaction is possible. In the *in vitro* assays, ACE is in solution and is freely accessible to the peptide, while in organ bath experiments ACE is present in the tissue as it is in the body. Furthermore, in the organ baths the substrate for ACE is Ang I, the *in vivo* substrate of ACE, while a synthetic substrate Hip-His-Leu is used in the *in vitro* assays.

In the organ baths, Ala-Val-Phe (5 mM) did not exert an inhibitory effect on the contraction evoked by Ang I. In contrast, in the presence of Ala-Val-Phe (5 mM), a slight increase in the contraction in response to the lowest concentration of Ang I was observed (Fig. 1B). This resulted in an Ang I pD_2 value in the presence of 5 mM Ala-Val-Phe which was slightly higher compared to the control (Table 3). In the presence of an Ala-Val-Phe concentration of 13.3 mM, which was tested in the organ baths twice, no inhibitory effect on the contraction evoked by Ang I was observed.

When the rat aortic rings were incubated with Val-Phe at a concentration of 5 mM, a rightward shift ($p < 0.0001$) compared with the control of the concentration–response curve to Ang I was observed (Fig. 1C); the same shift ($p = 0.84$) was seen with 10 mM of the dipeptide (data not shown). In contrast, the curve with 2 mM was equal ($p = 0.40$) to the control (data not shown). In Table 3, the Ang I pD_2 values and the maximum contraction are reported. A significant decrease (compared to control) of the Ang I pD_2 value and the maximal contraction is obtained for 5 and 10 mM Val-Phe. Then, the aortic rings were incubated with a concentration of 10 mM Val-Phe for 30 min before the contraction was evoked by Ang II to investigate a potential interaction of Val-Phe with the Ang II receptor. Val-Phe did not show a significant effect on the contractile response of the aortic ring to Ang II. The pD_2 values of Ang II in the presence of 0 and 10 mM Val-Phe, 9.29 ± 0.07 and 9.56 ± 0.19, respectively, are not significantly different. Similar to our previous study with Val-Tyr (5 mM), we again observed that after incubation with 10 mM Val-Phe, contraction declined in the presence of the highest concentrations of Ang II [12]. The fact that Val-Phe shifts the curve of Ang I but does not have any Ang II receptor blocking effect, leads to the conclusion that Val-Phe shows ACE inhibitory activity in isolated rat aorta.

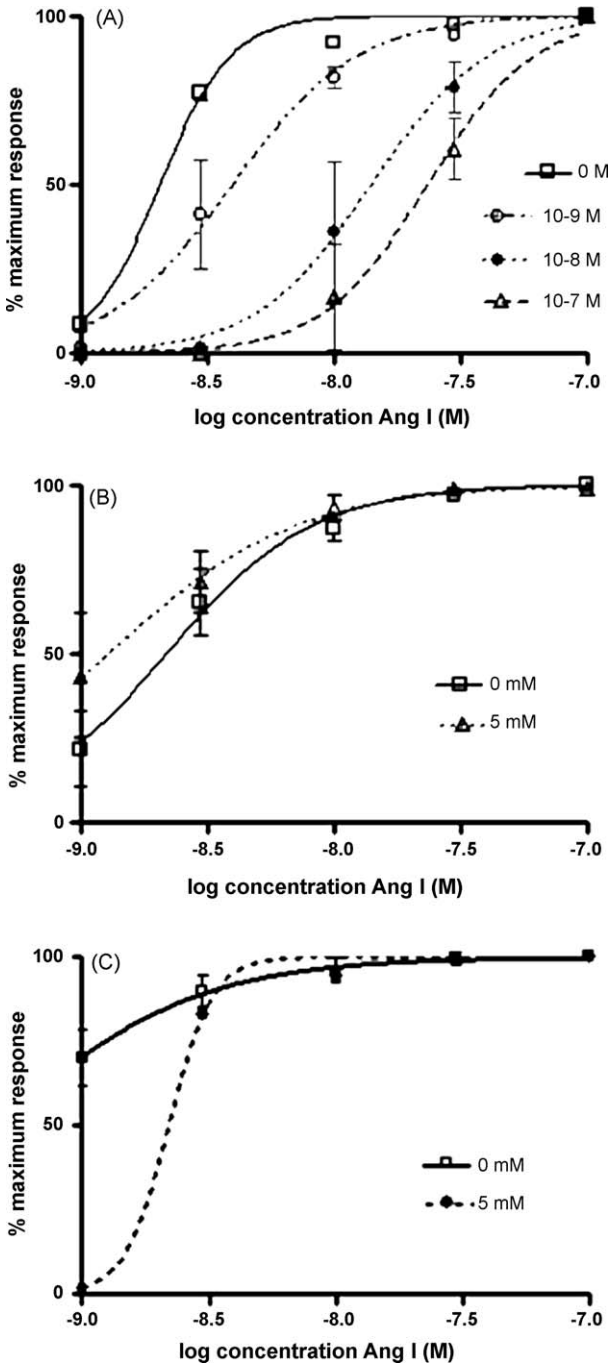

Fig. 1. Effect of lisinopril (A), Ala-Val-Phe (B) and Val-Phe (C) on the contraction evoked by angiotensin I in rat aortic rings. The effect was determined in the presence of NO synthase inhibitor N-nitro-L-arginine after preincubation of aortic rings in the presence of compound for 30 min. Values are expressed as a percentage of the maximum response. Experimental data ($n = 3$–6) were fitted by nonlinear regression and are expressed as mean ± SEM.

Table 3
Effect of lisinopril, Ala-Val-Phe and Val-Phe on the concentration–response curve to angiotensin I in rat aortic rings.

Compound	Concentration (mM)	Ang I pD_2	Maximal contraction (% of KCl contraction)
Lisinopril	0	8.68 ± 0.03 a	96.1 ± 2.3 a
	10^{-6}	8.40 ± 0.04 b	103.0 ± 4.5 a
	10^{-5}	7.85 ± 0.02 c	102.0 ± 5.0 a
	10^{-4}	7.63 ± 0.03 d	97.0 ± 3.0 a
Ala-Val-Phe	0	8.66 ± 0.03 a	93.7 ± 2.6 a
	5	8.89 ± 0.02 b	105.5 ± 1.7 b
Val-Phe	0	9.33 ± 0.03 a	99.1 ± 0.7 a
	2	9.29 ± 0.02 a	101.5 ± 0.9 a
	5	8.66 ± 0.04 b	87.6 ± 1.5 b
	10	8.64 ± 0.05 b	88.4 ± 1.9 b

Maximal contraction is expressed as % of the contraction evoked by 100 mM KCl solution in the same aortic ring. pD_2 values and maximum contraction were calculated by nonlinear regression of experimental data (log IC_{50} values were extrapolated from the concentration–response curves for the concentration of 0 and 2 mM Val-Phe). Per compound, the letters (a–d) within the same column indicate significant differences as determined by a comparison of fit ($p < 0.05$) ($n = 3$–6).

3.5. Antihypertensive effect in SHR

SHR is an accepted animal model to evaluate the antihypertensive activity of selected ACE inhibitory peptides or hydrolysates. In our study, the chemically synthesized peptides Ala-Val-Phe (5 mg/kg BW) and Val-Phe (5 mg/kg BW) were orally administrated by gavage to SHR. Although Ala-Val-Phe exerted a low *in vitro* ACE inhibitory activity, we decided to evaluate the *in vivo* antihypertensive activity as previous reports indicated a discordance between *in vitro* and *in vivo* activity [9,17]. A good example is that Tyr-Pro, identified from a yoghurt-like product (fermentation by *Lactobacillus helveticus*), exerted an antihypertensive activity between 2 and 8 h after oral administration at a dose of 1 mg/kg BW. However, the *in vitro* ACE inhibitory activity of Tyr-Pro was rather low with an IC_{50} value of 720 μM.

The basal value of blood pressure of the SHR used in this study was 188 ± 3 mm Hg. Fig. 2A shows the changes in SBP after single oral administration of Ala-Val-Phe. A significant decrease in SBP of 13 ± 3 mm Hg was measured at 4 h postadministration. At 6 h after gavage, a decrease in SBP was observed although this decrease was not significant ($p < 0.10$). The effect of Val-Phe on SBP is shown in Fig. 2B. Val-Phe exerts a maximum decrease in SBP of 19 ± 3 mm Hg at 6 h after gavage. A decrease in SBP was also obtained at 2, 4 and 8 h postadministration, but this decrease was not significant ($p < 0.10$). SBP returned to pretreatment value after 24 h. These results indicate that both Ala-Val-Phe and Val-Phe exert *in vivo* antihypertensive activity after single oral administration, with Val-Phe showing the highest activity.

3.6. Molecular docking

Docking experiments performed with the stable conformations of Val-Phe and Ala-Val-Phe offer a molecular basis for the inhibitory activity of the peptides on ACE, in this case tACE. tACE consists of a single polypeptide chain of 625 residues arranged in two subdomains: 1 (residues 1–292) and 2 (residues 293–623), around a central groove where the active site of the enzyme is located (Fig. 3A,B). The enzyme is predominantly built up from extended α-helices associated with a few short β-strands. tACE interacts with captopril *via* a network of six hydrogen bonds involving those amino acid residues usually involved in the binding of the dipeptide substrate. They correspond to Gln281, His353, Glu384, Lys511, Lys513 and Tyr520 (Fig. 3D). Three other residues (His383, His387 and Glu411) located in the vicinity

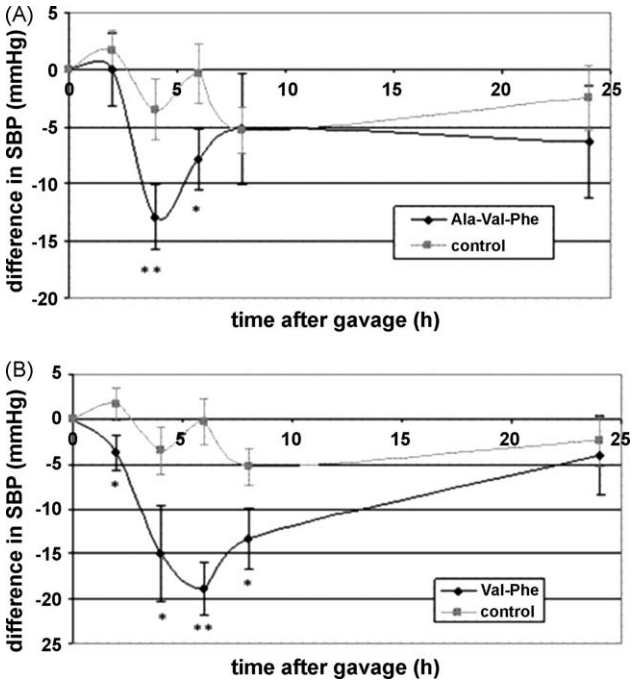

Fig. 2. Antihypertensive activity of Ala-Val-Phe (A) and Val-Phe (B) after single oral administration (5 mg/kg BW) in SHR. Changes in systolic blood pressure from zero time were expressed as mean ± SEM. *,** indicate significant differences against control (**$p < 0.05$; *$p < 0.1$) by *t*-test.

participate in the coordination of the catalytically active zinc ion. All these residues occur at the bottleneck connecting the two N- and C-chambers of the enzyme (Fig. 3C). Upon docking with Val-Phe, the active dipeptide readily binds to the substrate-binding site of tACE *via* a network of hydrogen bonds very similar to that found with the tACE drug inhibitors, e.g., captopril or lisinopril [3,4,23,27]. However, due to the orientation of both rings of the Phe residue of Val-Phe, which become too far (distance >3.5 Å) from Glu384 to create an additional hydrogen bond (Fig. 3E,F), the network of hydrogen bonds that connects the dipeptide to tACE is apparently weaker than that observed with, e.g., captopril. On the other hand, residue His353 creates two hydrogen bonds with the dipeptide and an additional stacking interaction occurs between the aromatic ring of Phe and His383A (Fig. 3F). The introduction of an additional Ala residue in the more bulky Ala-Val-Phe tripeptide prevents its binding to the substrate-binding site of tACE due to the occurrence of severe steric clashes (<1.0 Å) with the surrounding amino acid residues (results not shown). These docking results agree with the previously reported *in vitro* active (Val-Phe) and less active character (Ala-Val-Phe) of the tested peptides. It should be noted that the docking experiments were performed using tACE and not somatic ACE (sACE) which is the key enzyme for blood pressure regulation. However, tACE is almost identical to the C-terminal half of sACE, which has been demonstrated to be necessary and sufficient for blood pressure regulation [23]. Furthermore, the docking experiments only evaluate the binding of the peptides to ACE at the interaction site of captopril with ACE. Interactions of the peptides with other parts of ACE may possibly also lead to ACE inhibition.

Considering all the results reported in this paper, we could suggest that Ala-Val-Phe may exert its antihypertensive activity by releasing Val-Phe *in vivo*. In the *in vitro* assay, Ala-Val-Phe showed a low ACE inhibitory activity, moreover, no activity could be observed at tissue level in the organ baths. In both these assays, no enzymatic conversion of the tripeptide is possible as we showed that Ala-Val-Phe was stable against

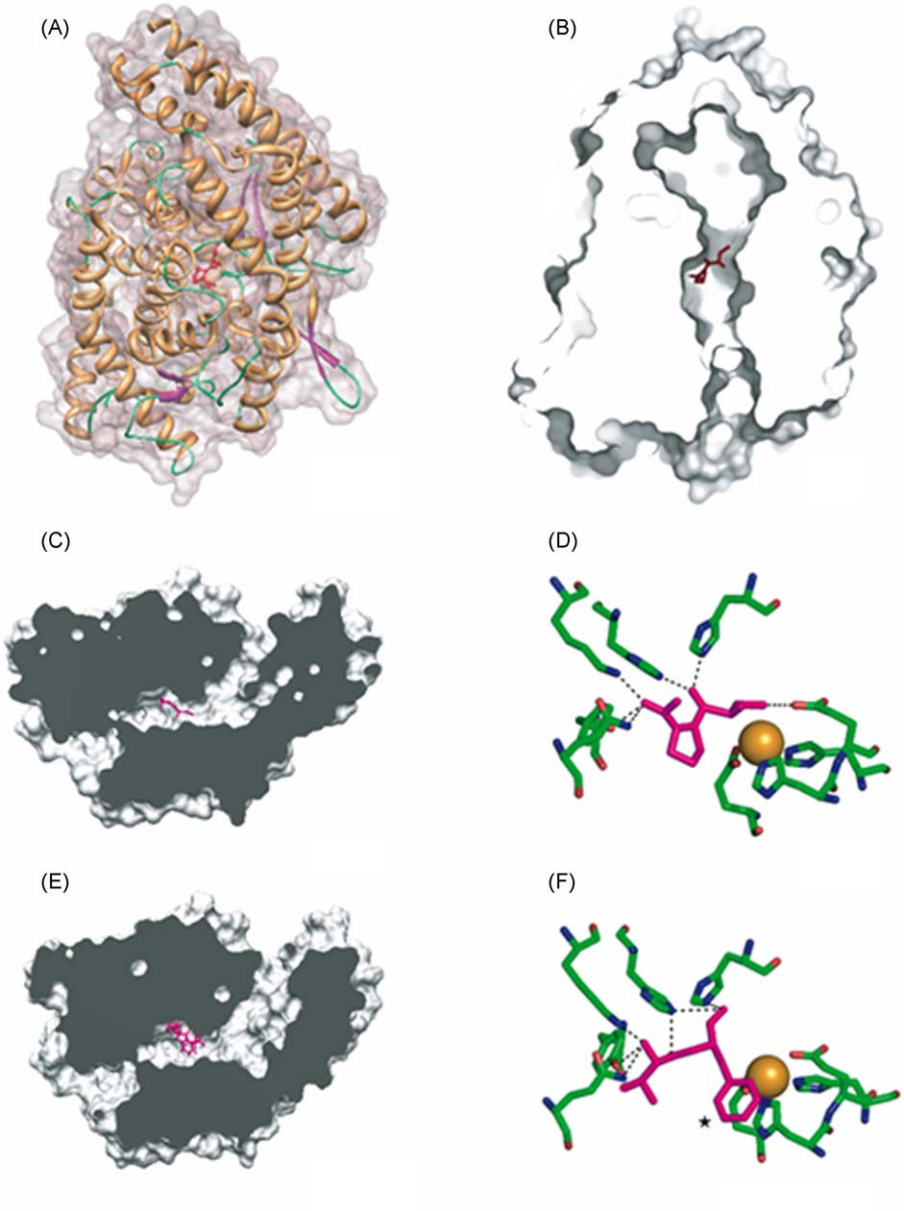

Fig. 3. (A) Ribbon diagram showing the overall three-dimensional structure of human tACE in complex with captopril (red ball and stick representation). (B) Clip showing the overall shape of the central groove of tACE. Captopril (red stick representation) occupies the bottleneck of the groove. (C and E) Clips of tACE showing the location of captopril (C) and dipeptide Val-Phe (E) represented in pink sticks at the bottleneck of the catalytic groove. (D and F) H-bond network anchoring captopril (D) and dipeptide Val-Phe (F) to the amino acid residues (Gln281, His353, Glu384, Lys511, His513, Tyr520) forming the catalytic site of tACE. Residues His383, His387 and Glu411 participate in the coordination of the Zn atom. Amino acids, captopril and dipeptide Val-Phe are represented by sticks and H-bonds are represented by black dotted lines. Stacking interactions are indicated by a star (★). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

degradation by ACE. However, *in vitro* digestion of Ala-Val-Phe by mucosal peptidases led to the release of Val-Phe, a more potent *in vitro* ACE inhibitor which also exerted ACE inhibitory activity in rat aortic rings. The docking experiments support the active character of Val-Phe as potential inhibitor of human ACE, while Ala-Val-Phe was shown to be inactive due to severe steric clashes by the additional Ala residue. *In vivo*, the experiments on SHR revealed an antihypertensive activity after single oral administration of Ala-Val-Phe and a slightly higher activity after oral administration of Val-Phe. Therefore, it can be postulated that after oral administration, a part of Ala-Val-Phe can be hydrolyzed by *in vivo* peptidases to release Val-Phe which is then responsible for the *in vivo* activity of Ala-Val-Phe. Nevertheless, some uncertainties of this hypothesis need to be mentioned. In our *in vitro* digestion of Ala-Val-Phe using mucosal peptidases only Val-Phe was released, however *in vivo* enzymes might also release Ala-Val which has been reported to show 15% ACE inhibitory activity at a concentration of 800 μM [12]. Furthermore, with the current experiments it cannot be ruled out that Ala-Val-Phe shows a direct effect on blood pressure regulation by ACE inhibition or by another antihypertensive mechanism. Further experiments, such as long term administration of the peptides to SHR can provide more information, as next to blood pressure, other factors can be evaluated, e.g., serum ACE activity, urine sodium concentration and organ weight (heart, liver, kidneys and lungs) [5,31,35].

To the best of our knowledge this is the first report of *in vivo* antihypertensive activity of peptides derived from insect protein. Our results indicate the potential of insect protein as source of bioactive peptides for functional foods or nutraceuticals. Further-

more, in accordance to previous literature, we indicated the importance of bioconversion/availability of bioactive peptides.

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