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Effect of Simulated Gastrointestinal Digestion on the Antihypertensive Properties of ACE-Inhibitory Peptides Derived from Ovalbumin

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Food-derived bioactive peptides with ACE-inhibitory properties are receiving special attention due to their beneficial effects in the treatment of hypertension. In this work we evaluate the impact of a simulated gastrointestinal digestion on the stability and activity of two bioactive peptides that derive from ovalbumin by enzymatic hydrolysis, YAEERYPIL and RADHPFL. These peptides possess in vitro ACE-inhibitory activity and antihypertensive activity in spontaneously hypertensive rats (SHR). The results showed that YAEERYPIL and RADHPFL were susceptible to proteolytic degradation after incubation with pepsin and a pancreatic extract. In addition, their ACE-inhibitory activity in vitro decreased after the simulated digestion. The antihypertensive activity on SHR of the end products of the gastrointestinal hydrolysis, YAEER, YPI, and RADHP, was evaluated. The fragments YPI and RADHP significantly decreased blood pressure, 2 h after administration, at doses of 2 mg/kg, but they probably did not exert their antihypertensive effect through an ACE-inhibitory mechanism. It is likely that RADHP is also the active end product of the gastrointestinal digestion of the antihypertensive peptides FRADHPFL (ovokinin) and RADHPF (ovokinin 2-7).

KEYWORDS: Ovalbumin; ACE-inhibitory peptides; gastrointestinal digestion; antihypertensive activity; spontaneously hypertensive rats

INTRODUCTION

Enzymatic hydrolysis of food proteins can release peptides able to exert different biological activities (*1*). Among the bioactive peptides known so far, those with angiotensin converting enzyme (ACE)-inhibitory properties are receiving special attention due to their potential beneficial effects in the treatment of hypertension. ACE is a multifunctional enzyme, located in different tissues, able to regulate several systems that affect blood pressure, as it is responsible for the generation of the vasopressor agent angiotensin II and for the inactivation of the vasodepressor agent bradykinin (*2*). The formation of ACE-inhibitory peptides by enzymatic hydrolysis of proteins from various food sources, including animal, milk, and vegetal proteins has been reported. Furthermore, in some studies, the antihypertensive properties of peptides with ACE-inhibitory activity were demonstrated in vivo using hypertensive animals and humans (*3*−*7*).

The physiological effects of ACE-inhibitory peptides depend on their ability to reach intact their target sites, which may involve absorption through the intestinal epithelium to get to the peripheral organs (*8*). The release of ACE-inhibitory peptides upon digestion of food proteins or protein fragments, as well as the resistance to digestion of known ACE-inhibitory sequences, have been tested in several in vitro studies that showed that proteolysis by gastrointestinal enzymes is an essential factor in determining ACE inhibitory activity (*9*−*11*).

In previous papers we showed that proteolysis of crude egg white with pepsin produced a hydrolysate with in vitro ACE-inhibitory activity and antioxidant properties that, therefore, could be potentially useful in the prevention or treatment of cardiovascular diseases (*12*, *13*). Several very active peptide sequences that derived from ovalbumin were identified using tandem mass spectrometry, peptide synthesis, and confirmation of the ACE-inhibitory and antioxidant properties of the synthetic fragments. Furthermore, the in vivo evaluation of the antihypertensive effects of the hydrolysate of egg white proteins and of some synthetic peptide sequences, with strong ACE-inhibitory activity in vitro, revealed that they efficiently reduced blood pressure in spontaneously hypertensive rats (SHR) (*14*). In particular, the synthetic peptides YAEERYPIL and RADHPFL exhibited a significant antihypertensive effect, lowering systolic blood pressure in a dose-dependent manner at minimum effective doses of around 2 mg/kg (*14*).

In this work we evaluated the impact of gastrointestinal digestion on the stability and activity of peptides YAEERYPIL

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and RADHPFL that possessed in vitro ACE-inhibitory activity and antihypertensive activity in SHR. Our aim was to further understand the in vivo effects and elucidate whether the intact sequences had physiological relevance in blood pressure regulation.

MATERIALS AND METHODS

Synthetic Peptides. Synthetic peptides YAEERYPIL, YAEER, YPI, FRADHPFL, RADHPFL, RADHPF, RADHP, and ADHP were obtained by conventional Fmoc solid phase synthesis with a 431 peptide synthesizer (Applied Biosystem Inc., Überlingen, Germany), and their purity was verified by RP-HPLC-MS/MS.

Simulated Gastrointestinal Digestion. The two-stage hydrolysis process was carried out according to Alting et al. (*15*) as described by Gómez-Ruiz et al. (*11*). Aqueous solutions of the synthetic peptides (2 mg/mL) were first hydrolyzed with pepsin (EC 3.4.4.1; 1:60 000, 3400 U/mg) (Sigma) (enzyme:substrate ratio of 1:50 w/w) at pH 2.0 and 37 °C for 90 min followed by hydrolysis with corolase PP (enzyme: substrate ratio of 1:50 w/w) (Röhm, Darmstadt, Germany) at pH 7.0 and 37 °C for 240 min. Corolase PP is a proteolytic enzyme preparation from the pig pancreas gland that, in addition to trypsin and chymotrypsin, contains numerous amino and carboxypeptidase activities. Hydrolyses were carried out in a thermally controlled incubator under continuous stirring (Unitron, Infors AG, Bottmingen, Switzerland). The reaction was stopped by heating at 95 °C for 10 min in a water bath, followed by cooling to room temperature. Each sample was stored at −20 °C until further analysis.

Analysis by On-line RP-HPLC-MS/MS. Identification of peptides in the hydrolysates obtained after simulation of the gastrointestinal digestion was performed on an Agilent HPLC system (Agilent Technologies, Waldbronn, Germany) connected on-line to an Esquire-LC quadrupole ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany). The HPLC was equipped with a quaternary gradient pumping system, an in-line degasser, a variable wavelength absorbance detector set at 214 nm, and an automatic injector (all 1100 Series, Agilent Technologies, Waldbronn, Germany). The column used in these experiments was a 250 mm × 4.6 mm Widepore C_{18} column (Bio-Rad, Richmond, CA). The injection volume was 50 μL. Solvent A was 0.37 mL/L TFA in milli-Q water and solvent B 0.27 mL/L TFA in acetonitrile. Peptides were eluted with a linear gradient of solvent B in A, from 2 to 10% in 15 min, 10 to 20% in 35 min, and 20 to 30% in 20 min. The flow rate was 0.8 mL/min. The flow was split post detector by placing a T-piece (Valco, Houston, TX) connected with a 75 μm ID peek outlet tube of an adjusted length to give approximately 20 μL/min of flow directed into the mass spectrometer via the electrospray interface. Nitrogen was used as nebulizing and drying gas and operated with an estimated helium pressure of 5×10^{-3} bar. The capillary was held at 4 kV. Spectra were recorded over the mass/charge (*m*/*z*) range 100−1500. About 25 spectra were averaged in the MS analyses and about 5 spectra in the MS(n) analyses. The signal threshold to perform auto MS(n) analyses was 5000, and the precursor ions were isolated within a range of 4.0 *m*/*z* and fragmented with a voltage ramp going from 0.35 to 1.4 V. Using Data Analysis (version 3.0; Bruker Daltoniks), the *m*/*z* spectral data were processed and transformed to spectra representing mass values. BioTools (version 2.1; Bruker Daltoniks) was used to process the MS(n) spectra and to perform peptide sequencing (*11*).

ACE-Inhibitory Activity. ACE-inhibitory activity of the purified peptides and the hydrolysates obtained by simulation of the gastrointestinal digestion was measured in vitro following the spectrophotometric assay described by Cushman and Cheung (*16*) with some modifications (*12*) as explained below. The substrate, hippuryl-histidyl-leucine (HHL) and angiotensin converting enzyme (ACE) from rabbit lung (EC 3.4.15.1) were purchased from Sigma. Testing solutions (40 μL) were incubated with 100 μL of 0.1 M borate buffer (pH 8.3) containing 5 mM HHL and 0.3 M NaCl and with 20 μL of ACE (2 mU) at 37 °C for 30 min. The reaction was stopped with 150 μL of 1 M HCl. The hippuric acid formed was extracted with ethyl acetate (1000 μL) and centrifuged at 1500*g* for 10 min, and 750 μL of the organic phase was evaporated. The residue was dissolved in 800 μL of distilled water, and the absorbance was measured at 228 nm. Triplicate tests were performed for each sample. Inhibitory activity was expressed as the concentration needed to inhibit 50% of ACE activity (IC_{50}). For this purpose, protein concentration was determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) using bovine serum albumin as standard.

Antihypertensive Activity in Spontaneously Hypertensive Rats (SHR). Male SHR, 17 to 20 weeks old, weighing 300−350 g (Charles River Laboratories Spain S.A.) were used in this study. The animals were maintained at a temperature of 23 °C with 12 h light/dark cycles and consumed tap water and a standard diet for rats (A04 Panlab, Barcelona, Spain) ad libitum during the experiments. The peptides were orally administered at a dose of 2 mg/kg by gastric intubation, between 9 and 10 a.m. Distilled water served as negative control, and captopril (50 mg/kg) (Sigma, USA), a known ACE inhibitor, served as positive control. All the products were administered in 1 mL of water. The systolic blood pressure (SBP) of the rats was measured by the tail cuff method (*14*), before administration and also 2, 4, 6, 8, and 24 h post-administration using an LE 5001 equipment (Letica, Hospitalet, Barcelona, Spain). Before the measurement, the rats were kept at 37 °C for 10 min to make the pulsations of the tail artery detectable. To guarantee the reliability of the measurements, the rats were accustomed to the procedure two weeks before beginning the experiments. Changes in SBP were calculated as the difference between the mean values of six measurements obtained before and after the administration. The results are expressed as mean values ± SEM for a minimum of 5 rats. These were analyzed by one-way ANOVA. Differences between the groups were assessed by the Bonferroni test and were considered significant when $P < 0.05$. All the above-mentioned experiments were performed as authorized for scientific research (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food).

RESULTS AND DISCUSSION

Simulated Gastrointestinal Digestion of YAEERYPIL and RADHPFL: Peptides Released and ACE-Inhibitory Activity. Previous studies have demonstrated the influence of in vitro gastrointestinal digestion on the formation and degradation of ACE-inhibitory activity from food proteins (*9*, *11*). However, the conditions of the in vitro process (enzyme preparation, temperature, pH, and incubation time) greatly influence the degree of proteolysis and the resultant ACE-inhibitory activity (*10*). In the present study, we simulated the in vivo conditions of protein digestion under physiological conditions. During fasting, the gastric pH is between 1.5 and 2.0 and pH of the overall small intestine is around 6.5. The half-emptying time for the stomach is 0.5−3 h, and the residence time in the duodenum and jejunum is 2−2.75 h and in the ileum 5−7 h (*17*). According to Vermeirssen et al. (*10*) a simulated digestion under physiological conditions provided an acceptable degree of proteolysis in the case of whey and pea proteins.

As previously reported, the peptide YAEERYPIL is a strong ACE inhibitor (IC_{50} = 5.4 μg/mL) that also exhibits a high radical scavenging activity (ORAC-FL value = 3.8 μmol of Trolox equiv/μmol of peptide) and delays LDL lipid oxidation induced by Cu^{2+} at a concentration around ∼0.16 mg/mg of LDL (*12*, *13*). **Figure 1a** shows the RP-HPLC chromatogram of YAEERYPIL after simulated gastrointestinal digestion. The peptide was totally hydrolyzed after incubation with pepsin and the pancreatic extract. The main fragments released were YAEER and the tripeptide YPI. A small proportion of YAEERYPI, which arose from the loss of C-terminal Leu, was also found, suggesting that this octapeptide was an intermediate proteolytic fragment. The peptide YPI was chemically synthesized and submitted to hydrolysis under gastrointestinal conditions. As expected, YPI remained intact (**Figure 1b**), suggesting that this tripeptide might be an end-product of gastrointestinal hydrolysis.

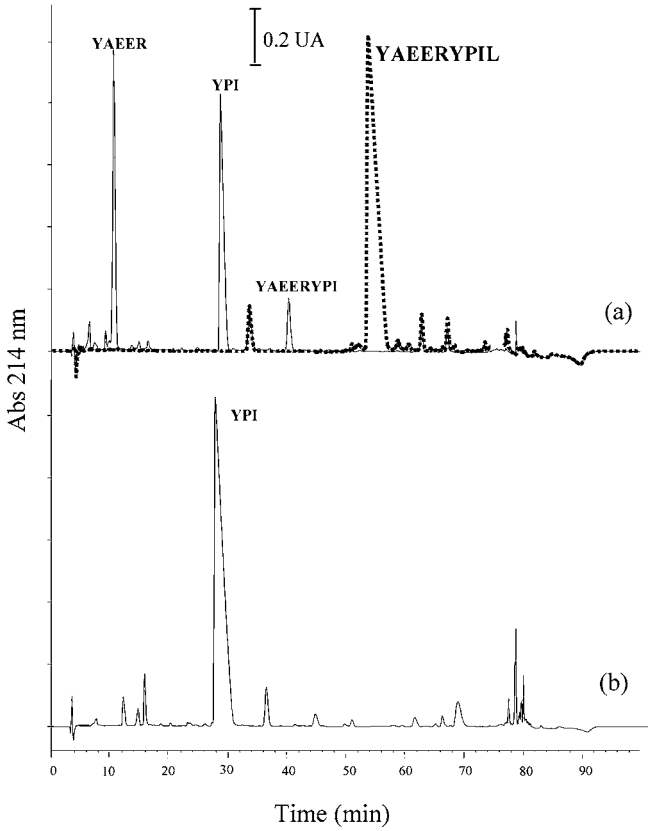

Figure 1. RP-HPLC chromatograms of the hydrolysates of synthetic peptides YAEERYPIL (**a**) and YPI (**b**) submitted to a simulated gastrointestinal digestion. The dotted line shows the RP-HPLC profile of YAEERYPIL before the digestion.

As shown in **Table 1**, the ACE-inhibitory activity of YAEERYPIL decreased by approximately 100-fold after simulated digestion. Looking at its degradation products, the synthetic peptide YAEER was not active as an ACE inhibitor (IC_{50} > 1000 μg/mL), which might be due to the presence of two dicarboxylic amino acids at the penultimate and antepenultimate positions, which negatively affect ACE inhibition (*18*). The second main fragment, YPI, was also a weak inhibitor, with an IC_{50} value higher than 1000 μg/mL. Research on the structure−activity relationship of ACE-inhibitory peptides pointed out that the C-terminal tripeptide region of the substrate significantly influences binding to ACE and that peptides containing hydrophobic amino acid residues in the C-terminal region, particularly Pro, display a high potency for inhibition. However, peptides with a Pro residue at the penultimate position may bind to ACE more weakly than those with Pro at the ultimate position (*19*). The sequence IPY found in Manchego cheese showed a lower IC_{50} (204.9 μM) (*11*), probably due to the presence of Tyr as the C-terminal residue.

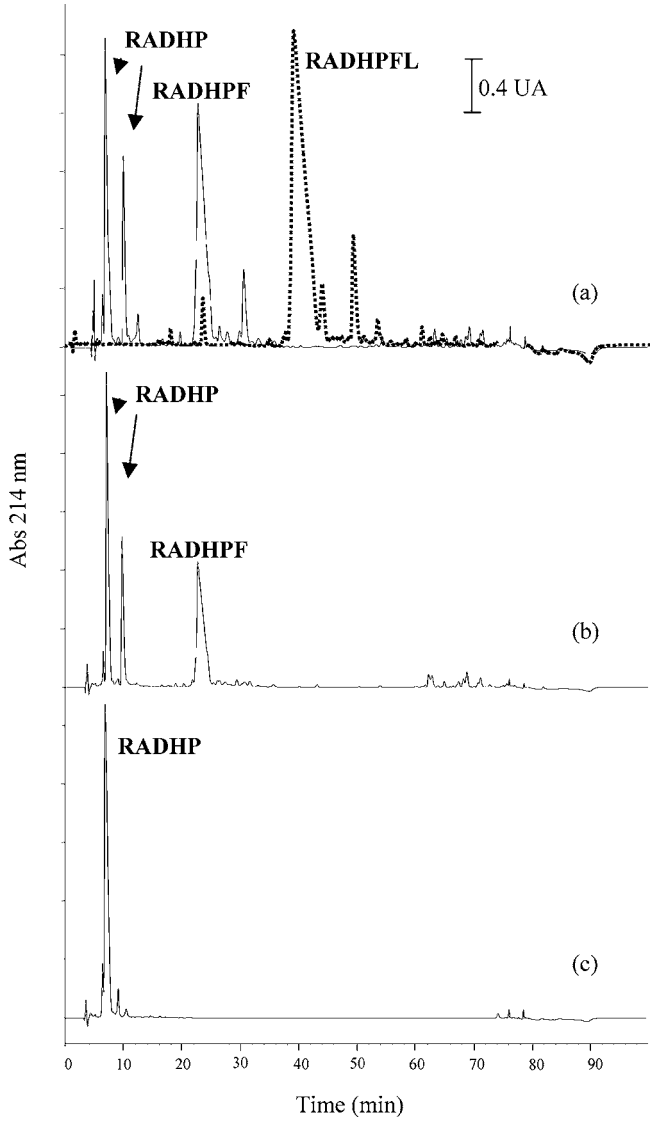

Figure 2. RP-HPLC chromatograms of the hydrolysates of synthetic peptides RADHPFL (**a**), RADHPF (**b**), and RADHP (**c**) submitted to a simulated gastrointestinal digestion. The dotted line shows the RP-HPLC profile of RADHPF before the digestion.

The second group of sequences studied were related to RADHPFL, a peptide with a high ACE-inhibitory activity (IC_{50} = 5.3 μg/mL) (*12*). When RADHPFL was subjected to a process mimicking gastrointestinal digestion, the main degradation products were RADHPF and RADHP (**Figure 2a**). Synthetic RADHPF partially resisted proteolysis with pepsin and the pancreatic extract but also gave RADHP as the main degradation product (**Figure 2b**). RADHP was not susceptible to the action of gastrointestinal enzymes (**Figure 2c**).

Table 1. Peptide Fragments Released and ACE-Inhibitory Activity (Means ± SEM) after Simulated Gastrointestinal Digestion of the Synthetic Peptides

sequence	degree of hydrolysis	fragments released after digestion	IC_{50} (μg/mL) before digestion	IC_{50} (μg/mL) after digestion
YAEERYPIL	hydrolyzed	YAEERYPI, YAEER, YPI	5.4 ± 0.8	446 ± 1.8
YPI	not hydrolyzed		>1000	>1000
YAEER	ND[a]	ND[a]	>1000	ND[a]
FRADHPFL	hydrolyzed	FRADHPF, RADHPF, ADHPF, RADHP	3.22 ± 0.9	90.21 ± 11.3
RADHPFL	hydrolyzed	RADHPF, RADHP	5.3 ± 1.02	521 ± 31.4
RADHPF	partly hydrolyzed	RADHPF, RADHP	382.5 ± 30.1	744.7 ± 9.2
RADHP	not hydrolyzed		153 ± 20.5	153 ± 20.5
ADHP	not hydrolyzed		>500	>500

[a] ND: not determined.

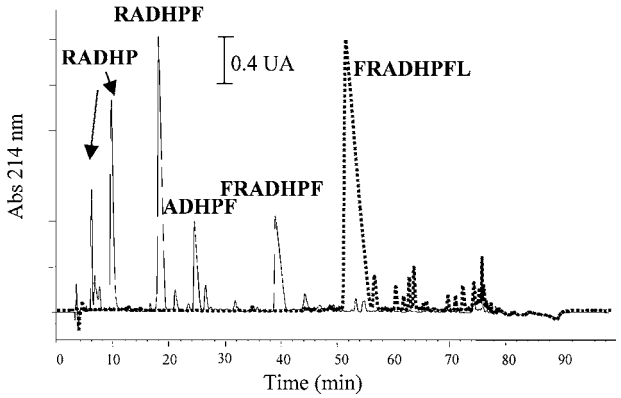

Figure 3. RP-HPLC chromatograms of the hydrolysate of synthetic peptide FRADHPFL submitted to a simulated gastrointestinal digestion. The dotted line shows the RP-HPLC profile of FRADHPFL before the digestion.

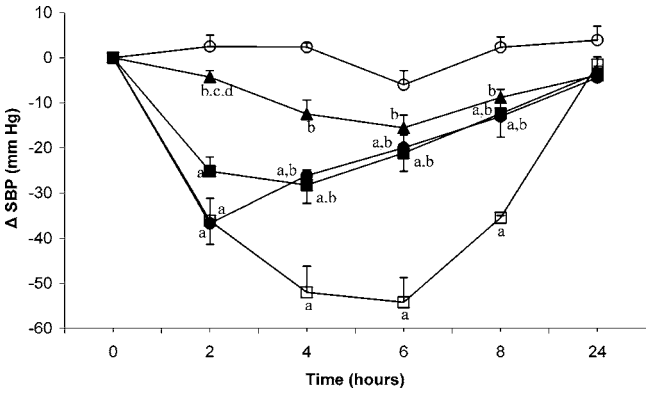

Figure 4. Decreases in SBP after the administration to SHR of 1 mL water (○), 50 mg/kg of captopril (□), 2 mg/kg of YAEER (▲), 2 mg/kg of YPI (●), and 2 mg/kg of RADHP (■). Data are expressed as mean ± SEM for a minimum of 5 animals. (a) $P < 0.05$ vs water. (b) $P < 0.05$ vs captopril. (c) $P < 0.05$ vs YPI. (d) $P < 0.05$ vs RADHP.

The peptide RADHPFL resembles the sequence FRADHPFL lacking the N terminus Phe residue. The octapeptide FRADHPFL, which possesses a high ACE-inhibitory activity (IC_{50} = 3.2 μg/mL) and a modest radical scavenging activity (ORAC-FL value = 0.128 μmol of Trolox equiv/μmol of peptide) (*12*, *13*), was named Ovokinin by Fujita et al. (*20*). Ovokinin exerts vasorelaxing effects in canine mesenteric arteries and significantly lowers the systolic blood pressure in SHR when orally administered in solution at a dose of 100 mg/kg, or at a dose of 25 mg/kg, in the form of an emulsion in 30% egg yolk (*20*, *21*). In view of the importance of FRADHPFL as an ACE-inhibitor and antihypertensive peptide, we also studied its stability to gastrointestinal proteinases. As shown in **Figure 3**, FRADHPFL was also hydrolyzed to RADHPF and RADHP, and it gave smaller amounts of FRADHPF and ADHPF.

Our results thus showed that the strong ACE-inhibitory peptides FRADHPFL and RADHPFL were digested in the gastrointestinal tract to the same end fragments. However, the ACE-inhibitory activity of both peptides considerably decreased upon digestion. In fact, the determination of the ACE-inhibitory properties of their proteolysis products, RADHPF and RADHP, revealed that they were weak inhibitors, with IC_{50} values of 382 and 153 μg/mL, respectively (**Table 1**). The peptide RADHPF had been obtained from a chymotryptic digest of ovalbumin by Matoba et al. (*22*) and named Ovokinin 2-7. Ovokinin 2-7 and its synthetic analogues RPFHPF and RPLKPW lowered blood pressure in SHR with minimal effective doses of 10, 1, and 0.1 mg/kg, respectively (*22*−*24*). According to these authors, the stronger activities of Ovokinin 2-7 derivatives were related to their higher resistance to proteases in the digestive tract (*24*).

It should be mentioned that the RP-HPLC-MS analyses of the hydrolysates of FRADHPFL, RADHPFL, and RADHPF revealed the presence of two peaks with the same *m*/*z* (595.5). Upon fragmentation by MS/MS they also presented the same fragmentation profile, coincident with that of RADHP, which suggested that both peaks corresponded to different conformations of the same peptide (**Figures 2a,b** and **3**). According to Gómez-Ruiz et al. (*25*), peptides having a Pro residue at the end of their sequence, such as DKIHP, might display different conformations that lead to multiple chromatographic peaks. Interestingly, these authors showed that the change of a *trans*- to a *cis*-form of Pro can cause significant changes in the inhibitor structure of the peptide and its interactions with the active site of ACE (*25*).

In Vivo Activity of the Peptides Resulting from the Simulated Gastrointestinal Digestion. The results obtained suggested that YAEERYPIL and RADHPFL were susceptible to proteolytic degradation during gastrointestinal digestion. Then, the possibility arises that their end fragments YAEER, YPI, and RADHP are active as antihypertensive components. This hypothesis was checked by performing in vivo studies in SHR.

As shown in **Figure 4**, YAEER exhibited a slight, but not significant, antihypertensive effect, while YPI and RADHP significantly decreased SBP when administered to SHR at doses of 2 mg/kg. The decrease in SBP was maximal 2−4 h after administration, and SBP returned to baseline after 24 h. In the case of the peptides YAEERYPIL and RADHPFL, maximum reductions in SBP (−31.6 ± 2.6 and −34.0 ± 1.6 mmHg, respectively) are achieved 6 h after oral administration of 2 mg/kg doses (*14*). This time lag may be explained by the time required for the enzymatic digestion in vivo of the parent peptides.

The amount of YPI and RADHP that was required to show antihypertensive effects was relatively small in comparison with other food-derived peptides. For instance, 4 h after the administration of 0.3 or 0.6 mg/kg of the potent ACE inhibitors IPP and VPP (IC_{50} = 5 and 9 μM), SBP of SHR decreased by 21.7 and 32.13 mmHg, respectively (*26*). ACE-inhibitory peptides derived from spinach Rubisco, with IC_{50} values from 0.6 to 4.2 μM, had an antihypertensive effect after oral administration to SHR at doses from 20 to 100 mg/kg (*27*). In addition, after 2 h, our peptides led to decreases in blood pressure in SHR comparable to that of 50 mg/kg of the synthetic ACE-inhibitor captopril, even if they were used at much lower doses (**Figure 4**). In fact, the antihypertensive potency of YPI and RADHP was very high in comparison with their in vitro efficacy to inhibit ACE (**Table 1**). A possible explanation is that these peptides need further activation into in vivo active compounds by intestinal brush-border or plasma peptidases. Thus, the tripeptide YPI might be further hydrolyzed to the dipeptide YP that, in agreement with Yamamoto et al. (*28*), significantly reduces blood pressure in SHR, although through an unknown mechanism. Similarly, the possibility also arises that the inhibition of ACE might not be the only mode of action of YPI and RADHP.

Increasing new evidence is being provided that a mechanism other than ACE inhibition can be involved in the blood pressure lowering effect exerted by many food-derived peptides (*8*, *29*). Some studies have highlighted the existence of vasorelaxant peptides that exert their effects through the stimulation of opioid receptors (*30*). Furthermore, certain peptides exhibited a direct

or indirect action on vascular smooth muscles (*31*, *32*). In fact, ovokinin, (FRADHPFL) and ovokinin 2-7 (RADHPF) have been reported to lower blood pressure through different modes of vasorelaxing activity (*22*, *24*, *33*). In addition, strong experimental evidence indicates that oxidative stress and associated oxidative damage are mediators in cardiovascular pathologies, and thus antioxidant activity can also be responsible for hypotensive effects (*34*). In this respect, it should be noted that YPI has a radical scavenging activity of 1.570 μmol Trolox equiv/μmol (*13*). Further studies in vivo are being conducted to clarify the mechanisms implicated in the antihypertensive activity of these products.

ABBREVIATIONS USED

ACE, angiotensin-converting enzyme; ANOVA, analysis of variance; IC_{50}, protein concentration needed to inhibit 50% of ACE activity; ORAC-FL, oxygen radical absorbance capacity fluorescein assay; LDL, low-density lipoprotein; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RP-HPLC, reversed phase high-performance liquid chromatography; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; SEM, standard error of the mean; TFA, trifluoroacetic acid.

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