JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 6721–6727 6721
DOI:10.1021/jf100899u

Antihypertensive Properties of Lactoferricin B-Derived Peptides

Pedro Ruiz-Giménez,[†] Aida Ibáñez,[†] Juan B. Salom,[‡,§,∥] Jose F. Marcos,[⊥] Jose Javier López-Díez,[†] Salvador Vallés,[†] Germán Torregrosa,[‡,§,∥] Enrique Alborch,[‡,§,∥] and Paloma Manzanares*,[†]

[†]Departamento de Biotecnología de Alimentos, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas, Burjassot, Valencia, Spain, [‡]Centro de Investigación, Hospital Universitario 'La Fe', Valencia, Spain, [§]Departamento de Fisiología, Universidad de Valencia, Valencia, Spain, [∥]Unidad Mixta de Investigación Cerebrovascular, Fundación Investigación Hospital La Fe−Universidad de Valencia, Valencia, Spain, and [⊥]Departamento de Ciencia de los Alimentos, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas, Burjassot, Valencia, Spain

A set of eight lactoferricin B (LfcinB)-derived peptides was examined for inhibitory effects on angiotensin I-converting enzyme (ACE) activity and ACE-dependent vasoconstriction, and their hypotensive effect in spontaneously hypertensive rats (SHR). Peptides were derived from different elongations both at the C-terminal and N-terminal ends of the representative peptide $LfcinB_{20-25}$, which is known as the LfcinB antimicrobial core. All of the eight LfcinB-derived peptides showed in vitro inhibitory effects on ACE activity with different IC_{50} values. Moreover, seven of them showed ex vivo inhibitory effects on ACE-dependent vasoconstriction. No clear correlation between in vitro and ex vivo inhibitory effects was found. Only $LfcinB_{20-25}$ and one of its fragments, F1, generated after a simulated gastrointestinal digestion, showed significant antihypertensive effects in SHR after oral administration. Remarkably, F1 did not show any effect on ACE-dependent vasoconstriction in contrast to the inhibitory effect showed by $LfcinB_{20-25}$. In conclusion, two LfcinB-derived peptides lower blood pressure and exhibit potential as orally effective antihypertensive compounds, yet a complete elucidation of the mechanism(s) involved deserves further ongoing research.

KEYWORDS: Milk-derived peptides; lactoferrin; LfcinB-derived peptides; hypertension; ACE inhibition; ACE-dependent vasoconstriction; SHR; bioavailability; mechanism of action

INTRODUCTION

In recent years, interest in bioactive peptides with antihypertensive effects has grown as an alternative to drugs in the control of systemic blood pressure and prevention of associated cardiovascular disease events. Many studies have been performed on antihypertensive peptides derived from food proteins, including both animal- and plant-derived peptides. Some of them have shown in vivo hypotensive effects in animal models and in humans (*1*, *2*). This biological function seems to be related to the inhibition of angiotensin I-converting enzyme (ACE), a key enzyme in the renin−angiotensin system, which plays an important role in the regulation of systemic blood pressure. ACE hydrolyzes both the inactive angiotensin I into vasoconstrictor angiotensin II and the vasodilator bradikinin into an inactive peptide (*3*).

*Corresponding author. Departamento de Biotecnología de Alimentos, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas, P.O. Box 73, 46100 Burjassot, Valencia, Spain. Tel: 34-96-3900022. Fax: 34-96-3636301. E-mail: pmanz@iata.csic.es.

Most of the ACE inhibitory peptides share common structural features: they are short in length, and their potency is strongly influenced by their C-terminal tripeptide sequence, which usually contains hydrophobic amino acids and also proline, lysine, or arginine residues (*2*). Quantitative structure−activity relationship modeling of ACE inhibitory peptides has been described (*4*−*6*), and the relationship between structure and intestinal stability and permeability has been reported recently (*7*). Although these studies have permitted the generation of numerous ACE inhibitors, the relationship between in vitro potency and functional in vivo effects is not always substantiated. Potential explanations to this discrepancy are related to the bioavailability of peptides after oral administration or the possibility that peptides exert a hypotensive effect by mechanisms other than ACE inhibition (*8*).

Bovine milk proteins are a good source of ACE inhibitory peptides (*9*, *10*). Antihypertensive peptides generated from casein (*11*) as well as whey protein derived peptides (*12*) have been deeply studied. Bovine lactoferrin (LF), a minor component of milk whey, and its derived peptide lactoferricin B (LfcinB) have received considerable attention due to the diverse range of biological effects exhibited, including antimicrobial, antiviral,

Published on Web 05/06/2010

Table 1. Amino Acid Sequences of Peptides Used in This Study

peptide[a]	sequence
$LfcinB_{17-32}$	Ac-FKCRRWQWRMKKLGAP-NH_2
$LfcinB_{17-31}$	Ac-FKCRRWQWRMKKLGA-NH_2
$LfcinB_{20-25}$	Ac-RRWQWR-NH_2
$LfcinB_{19-25}$	Ac-CRRWQWR-NH_2
$LfcinB_{18-25}$	Ac-KCRRWQWR-NH_2
$LfcinB_{17-25}$	Ac-FKCRRWQWR-NH_2
$LfcinB_{17-24}$	Ac-FKCRRWQW-NH_2
$LfcinB_{17-22}$	Ac-FKCRRW-NH_2
$LfcinB_{22-23}$-F1	WQ
$LfcinB_{21-23}$-F2	RWQ

[a] All of the peptides were acetylated at the N-terminus (Ac) and amidated at the C-terminus (NH_2) with the exception of $LfcinB_{22-23}$-F1 and $LfcinB_{21-23}$-F2.

antioxidant, anticarcinogenic, and immunomodulatory activities (*13*, *14*). In contrast, there is scarce information about the possible antihypertensive activity of LF derived peptides. To date, only one ACE inhibitory peptide (LRPVAA) isolated from a pepsin LF hydrolysate has shown in vivo hypotensive activity in spontaneously hypertensive rats (SHR) after intravenous injection (*15*).

In previous works, we demonstrated inhibitory effects on ACE activity and ACE-dependent vasoconstriction of $LfcinB_{17-31}$ (FKCRRWQWRMKKLGA), a LfcinB-derived peptide representative of the antimicrobial motif of LF, and also of a LF pepsin hydrolysate using both in vitro and ex vivo functional assays (*16*, *17*). The objective of the present study was to further investigate the antihypertensive properties of the LF antimicrobial motif by characterizing a set of peptides derived from different elongations both at the C-terminal and N-terminal ends of $LfcinB_{20-25}$ (RRWQWR), known as the active core of LfcinB (*18*). We assessed their inhibitory effects on ACE activity and ACE-dependent vasoconstriction, and their hypotensive effect in SHR. Additionally, their in vitro stability against simulated gastrointestinal digestion and brush border peptidases was evaluated. Finally, the identification and functional characterization of two potential hypotensive $LfcinB_{20-25}$ fragments obtained after simulated digestion were also carried out.

MATERIALS AND METHODS

Materials. Angiotensin I-converting enzyme (ACE, EC 3.4.15.1) from porcine kidney, captopril, pepsin, and rat intestinal acetone powder were purchased from Sigma (Madrid, Spain). Angiotensin I and angiotensin II were provided by Calbiochem Co. (La Jolla, CA). Corolase PP (porcine pancreatic extract) was from AB Enzymes (Darmstadt, Germany).

Peptide Synthesis and Identification. Peptides (**Table 1**) were purchased at >90% purity from GenScript Corporation (Piscataway, NJ). Stock solutions of each peptide were prepared at 1 mM concentration in 5 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS) pH 7 buffer and stored at −20 °C. Peptide concentrations were determined by measuring the absorbance at 280 nm ($\varepsilon^{280} = 5600\ M^{-1}\ cm^{-1}$ for the W residue).

Fragments derived from $LfcinB_{20-25}$ were identified by RP-HPLC-MS/MS (ProteoRed, Proteomics Facility, Centro de Investigación Principe Felipe, Valencia, Spain).

In Vitro Assays of ACE Inhibitory Activity. Peptides were assayed in vitro for their capacity to inhibit the ACE activity using angiotensin I as substrate and quantifying the reaction product angiotensin II by RP-HPLC (*19*) with modifications as previously described (*16*).

Potency of the peptide inhibitory effect on ACE was expressed by using the IC_{50}, defined as the peptide concentration expected to inhibit 50% of the ACE activity, and the value for each experiment was calculated by nonlinear regression of the experimental data to a four-parameter logistic curve using SigmaPlot, version 10.0 (SPSS Inc., Chicago, IL).

Animals and Animal Welfare. Rabbits and rats were housed in temperature-controlled rooms (23 °C) with 12 h light/dark cycles and consumed tap water and standard diets ad libitum. Because of the stress associated with transportation, at least a one week period of acclimatization was allowed before the assays were carried out, as recommended (*20*).

Experimental procedures conformed to the Spanish legislation on Protection of Animals Used for Experimental and other Scientific Purposes and to the Directives of the European Community on this subject. Specifically, experimental protocols were refined to minimize any adverse effects for each individual animal, and the number of animals was reduced to the minimum consistent with achieving the scientific objectives of the study.

Twelve male New Zealand White rabbits (Technology Transferring Center, Polytechnic University of Valencia, Spain), weighing 2.5−3 kg, were euthanized by injecting 25 mg/kg sodium thiopental (Tiobarbital Braun, B. Braun Medical, Jaén, Spain) and 1.5 mL of 10 mM KCl solution through the ear vein. A midline throat incision provided access to both common carotid arteries, which were dissected free and cut in four 4 mm long segments. Twenty-nine male SHR weighing 300−350 g were used (Charles River Laboratories Spain, S.A).

Ex Vivo Functional Assay of Inhibitory Effect on ACE. For computer-assisted isometric tension recording, the arterial segments were mounted in an organ bath containing Ringer−Locke solution (120 mM NaCl, 5.4 mM KCl, 2.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25 mM $NaHCO_3$, and 5.6 mM glucose) as previously described (*17*).

The contractile capacity of every arterial segment was assessed by exposure to 50 mM KCl Ringer−Locke solution (NaCl was replaced with an equimolar amount of KCl). Carotid arteries contracting less than 2 g were discarded. Then, every arterial segment was challenged with a single concentration (1 μM) of angiotensin I. After washing out, each arterial segment was subjected to one of the following protocols: a second challenge to angiotensin I to check for response reproducibility or preincubation (20 min) with one of the LfcinB-derived peptides (20 μM), and a second challenge to angiotensin I to check for their effect on angiotensin I-induced contraction. This was a suitable protocol for checking peptide effects on captopril-sensitive ACE activity mediating vasoconstriction to angiotensin I (*16*).

In Vivo Assay of Antihypertensive Effect. Indirect measurement of systolic blood pressure (SBP) in awake restrained rats was carried out by the noninvasive tail-cuff method (*21*) using computer-assisted NIPREM 645 equipment (Cibertec, Madrid, Spain). Before the measurements, rats were kept at 37 °C during 15 min to make the pulsations of the tail artery detectable. The LfcinB-derived peptides were orally administered by gastric intubation at a dose of 10 mg/kg in 1 mL of physiological saline. The SBP was measured before peptide intake (zero time) and 1, 2, 3, 4, and 24 h after intake. Physiological saline alone (1 mL) and captopril (50 mg/kg) served as negative and positive controls, respectively. Each value of SBP was obtained by averaging three consecutive and successful measurements without disturbance of the signal. Changes in SBP were calculated as the difference between the averaged values of measurements obtained before and after peptide administration.

In Vitro Simulated Gastrointestinal Digestion and Stability Experiments. Peptides were subjected to a two-stage simulated gastrointestinal digestion process (*22*). Pepsin (0.2 g) was added to aqueous solutions of LfcinB-derived peptides (10 mL; 1 mM) adjusted at pH 2.0 using 1 N HCl and incubated at 37 °C. After 90 min, the pH was adjusted to 7.5 adding 10 mL of 0.4 M sodium phosphate buffer at pH 7.5. Corolase PP, a proteolytic enzyme preparation that contains trypsin, chymotrypsin, and amino and carboxypeptidase activities, was added (0.2 g), and the sample was further incubated at 37 °C 150 min. For stability experiments, the digestion was continued by a brush border phase (*23*) by adding rat intestinal acetone powder (0.8 mg) to the sample followed by further incubation at 37 °C for 60 min. The reaction was stopped by heating at 80 °C for 10 min in a water bath, followed by cooling at room temperature. After every stage, aliquots were taken to independently analyze each treatment by RP-HPLC, as specified above. Each sample was stored at −20 °C until further analysis.

RESULTS

In Vitro ACE Inhibitory Activity of LfcinB-Derived Peptides. In a previous work, we found no significant difference in in vitro

Table 2. Angiotensin I-Converting Enzyme Inhibitory Activity of LfcinB-Derived Peptides

peptide	IC_{50} (μM)[a]
$LfcinB_{17-32}$	11.0 ± 1.5 (4) b
$LfcinB_{17-31}$[b]	25.5 ± 2.3 (4) c
$LfcinB_{20-25}$	32.0 ± 4.9 (3) c
$LfcinB_{19-25}$	2.3 ± 0.1 (3) a
$LfcinB_{18-25}$	5.8 ± 0.2 (3) a,b
$LfcinB_{17-25}$	2.9 ± 0.6 (7) a
$LfcinB_{17-24}$	10.5 ± 0.6 (3) b
$LfcinB_{17-22}$	26.7 ± 1.9 (4) c

[a] Inhibitory potency is expressed as IC_{50}, and data are the mean ± SEM of (n) independent experiments. Data with the same letter are not significantly different, $P > 0.05$. One way ANOVA followed by Student−Newman−Keul's tests. [b] Ref *16*.

ACE inhibitory activity between $LfcinB_{20-25}$ and $LfcinB_{17-31}$, tested at a concentration of 20 μM, suggesting that the elongation of the $LfcinB_{20-25}$ sequence has no effect on ACE inhibition (*16*). To further support this conclusion as well as analyze the effect of sequence extension on the bioavailability and in vivo antihypertensive properties, we have designed a set of peptides derived from different elongations both at the C-terminal and N-terminal ends of $Lfcin_{20-25}$ (**Table 1**). **Table 2** shows the ACE inhibitory activity of peptides. Confirming our previous results, no differences in IC_{50} values were found between $LfcinB_{20-25}$ and $LfcinB_{17-31}$. Interestingly, different elongations of $LfcinB_{20-25}$ provoked differences in inhibitory potency. The higher potency as indicated by lower IC_{50} values corresponded to peptides $LfcinB_{19-25}$, $LfcinB_{17-25}$, and $LfcinB_{18-25}$, derived from elongations at the $LfcinB_{20-25}$ N-terminal end. However, the IC_{50} values for peptides $LfcinB_{17-25}$, $LfcinB_{17-24}$, and $LfcinB_{17-22}$ showed the importance of the R residue at the C-terminal position, in the context of the sequence studied. Remarkably, the addition of a P residue at the C-terminal end of $LfcinB_{17-31}$ provoked a 2-fold increase in the inhibitory potency of the resultant $LfcinB_{17-32}$.

Ex Vivo Inhibitory Effect of LfcinB-Derived Peptides on ACE-Dependent Vasoconstriction. We have previously reported the inhibitory effect of $LfcinB_{17-31}$ (20 μM) on angiotensin I-induced contraction of the rabbit carotid artery (control, 86 ± 3%, n = 27; $LfcinB_{17-31}$, 68 ± 6%, n = 10) (*16*). In the present study, challenge of the arterial segments with depolarizing solution (KCl 50 mM) induced contractions averaging 3862 ± 136 mg (n = 88). Angiotensin I (1 μM) induced phasic, transient contractions almost completely reproducible (89 ± 3%, n = 19) in two consecutive challenges to the same arterial segment. Preincubation (20 min) with LfcinB-derived peptides (20 μM) induced different inhibitory effects on angiotensin I-induced contractions (representative recordings are shown in **Figure 1**). As summarized in **Table 3**, all of the peptides tested, with the exception of $LfcinB_{19-25}$, induced significant inhibitions when compared to that of the control. As a general trend, there was no correlation between the in vitro potency of LfcinB derivatives and their effect on ACE-dependent vasoconstriction (compare **Tables 2** and **3**).

In Vivo Antihypertensive Effect of LfcinB-Derived Peptides in SHR. The average SBP for all measurements carried out in SHR from the 10 experimental groups before treatment intake (zero time) was 202 ± 2 mmHg (n = 79). Changes in SBP 1 h postoral administration (10 mg/kg) for all of the peptides tested are shown in **Figure 2**. $LfcinB_{20-25}$ was the only peptide showing significant antihypertensive effect ($P < 0.01$), although $LfcinB_{17-31}$ also led to a slight, but not significant, decrease in SBP. The time-course of the hypotensive effect of $LfcinB_{20-25}$ is shown in **Figure 3A**. The decrease in SBP observed for $LfcinB_{20-25}$ was less pronounced than that induced by captopril (50 mg/kg). The decrease was maximal 1 h after administration (−16.7 ± 3.2 mmHg), and SBP returned to baseline after 4 h, whereas the hypotensive effect of captopril lasted 24 h.

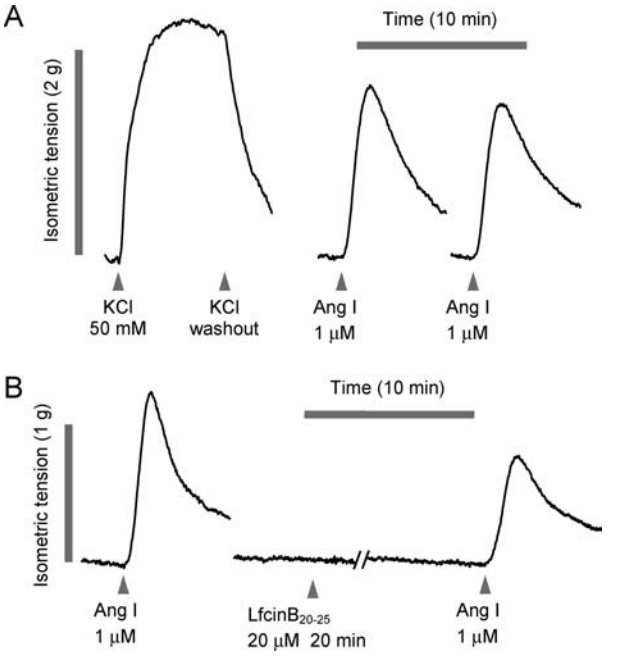

Figure 1. Contraction of carotid artery segments. (**A**) Effect of high-KCl to check for tissue viability and reproducible vasoconstriction to angiotensin I. (**B**) Effect of $LfcinB_{20-25}$ on angiotensin I-induced contraction.

Table 3. Effects of LfcinB-Derived Peptides on ACE-Dependent, Angiotensin I-Induced Arterial Contractions

peptide[a]	contraction peak (%)[b]
control	89 ± 3 (19)
$LfcinB_{17-32}$	71 ± 2 (6)[d]
$LfcinB_{20-25}$	63 ± 3 (5)[d]
$LfcinB_{19-25}$	86 ± 3 (7)
$LfcinB_{18-25}$	67 ± 3 (6)[d]
$LfcinB_{17-25}$	66 ± 2 (10)[d]
$LfcinB_{17-24}$	73 ± 4 (6)[c]
$LfcinB_{17-22}$	64 ± 1 (7)[d]

[a] Final concentration in the assay was 20 μM. [b] Second contraction response to angiotensin I is expressed as the percentage of the first contraction in the same arterial segment. Data are the mean ± SEM from (n) arterial segments. [c] Significantly different from the control, $P < 0.05$. [d] Significantly different from the control, $P < 0.01$. One-way ANOVA followed by Dunnett's multiple comparison tests.

Simulated Gastrointestinal Digestion and Intestinal Stability of LfcinB-Derived Peptides. In order to investigate the cause(s) for the lack of in vivo antihypertensive effect of most of the LfcinB-derived peptides, they were subjected to a hydrolysis process which simulates physiological digestion due to gastric and pancreatic enzymes and brush border peptidases.

The in vitro ACE inhibitory activities of the resulting digests were first determined. Most of the peptides showed IC_{50} values higher than 100 μM after digestion. Only digests of $LfcinB_{17-31}$, $LfcinB_{19-25}$, and $LfcinB_{17-24}$ showed IC_{50} values of 20.5 ± 5.4, 5.3 ± 0.4, and 18.7 ± 2.5 μM (n = 3), respectively, in the range of those obtained before digestion (see **Table 2**). The analysis of digests by RP-HPLC (data not shown) showed that pepsin treatment did not affect any of the peptides tested, whereas Corolase hydrolyzed all of them into several peptide fragments. $LfcinB_{17-31}$, $LfcinB_{19-25}$, and $LfcinB_{17-24}$ were only partly hydrolyzed (approximately 50% of the initial concentration of

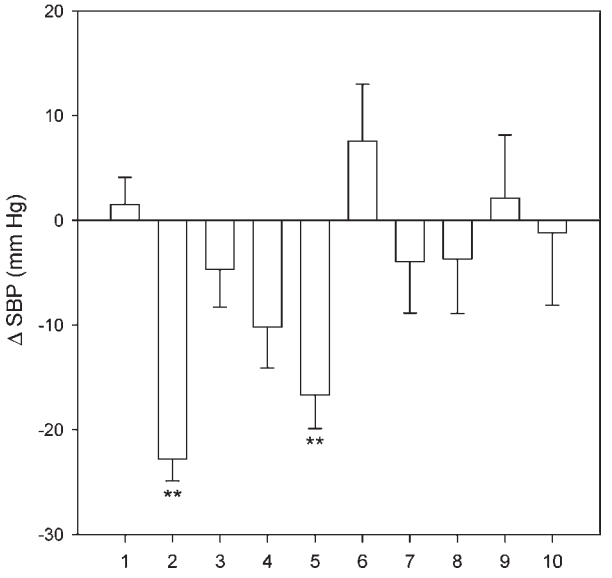

Figure 2. Changes in systolic blood pressure (SBP) 1 h postoral administration of captopril (50 mg/kg) and LfcinB-derived peptides (10 mg/kg) to SHR. Pressure changes are expressed in absolute values (mm Hg), and data bars are expressed as the mean ± SEM for a minimum of four animals ($n = 4-6$); $^{**}P < 0.01$ vs control group (one-way ANOVA followed by Dunnett's multiple comparison tests). 1, saline; 2, captopril; 3, $LfcinB_{17-32}$; 4, $LfcinB_{17-31}$; 5, $LfcinB_{20-25}$; 6, $LfcinB_{19-25}$; 7, $LfcinB_{18-25}$; 8, $LfcinB_{17-25}$; 9, $LfcinB_{17-24}$; 10, $LfcinB_{17-22}$.

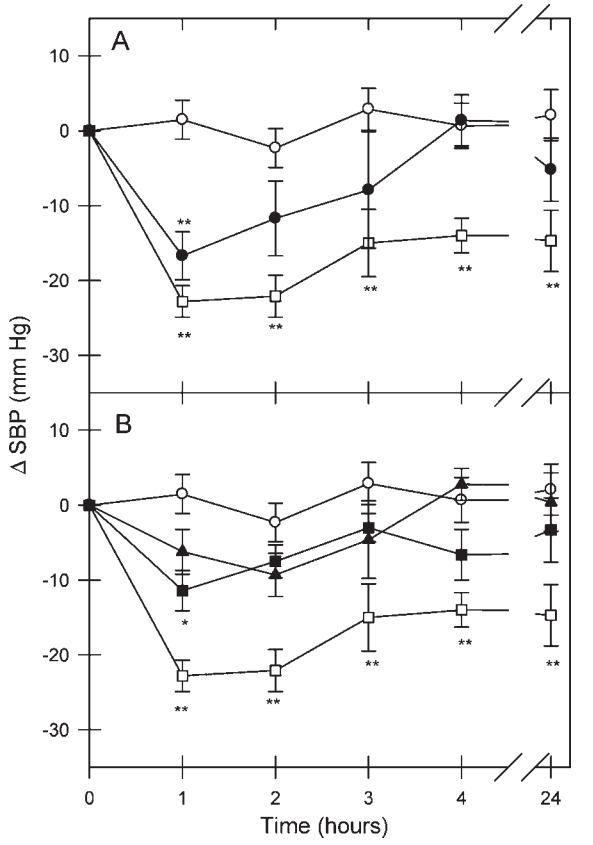

Figure 3. Time course of systolic blood pressure (SBP) changes after oral administration of physiological saline alone (○), captopril (□, 50 mg/kg) and LfcinB-derived peptides (10 mg/kg) to SHR. **Panel A**: (●), $LfcinB_{20-25}$ (Ac-RRWQWR-NH_2. **Panel B**: (■), $LfcinB_{22-23}$-F1 (WQ); (▲), $LfcinB_{21-23}$-F2 (RWQ). Pressure changes are expressed in absolute values (mm Hg), and data are expressed as the mean ± SEM for a minimum of four animals ($n = 4-6$); $^{*}P < 0.05$ vs control group and $^{**}P < 0.01$ vs control group (one-way ANOVA followed by Dunnett's multiple comparison tests).

the input peptide), whereas the rest of the LfcinB derivatives were almost completely hydrolyzed (hydrolysis > 90%). No significant differences between peptide profiles after the Corolase stage and intestinal peptidase treatment were observed.

Identification and Functional Characterization of $LfcinB_{20-25}$ Fragments Released by Simulated Digestion. Although the $LfcinB_{20-25}$ digest had no significant in vitro activity (IC_{50} > 100 μM), this peptide showed a hypotensive effect in vivo (**Figure 3**) and was completely hydrolyzed into several peptide fragments during the simulated physiological digestion. These facts prompted us to identify and characterize the ex vivo and in vivo effects of the main peptidic fragments released by the action of the digestive enzymes.

Figure 4 shows the peptide profile after simulated digestion and the identified fragments F1 (WQ) and F2 (RWQ). Neither F1 nor F2 (20 μM) showed significant effect on ACE-dependent, angiotensin I-induced vasoconstriction (control, 93 ± 3%, $n = 8$; F1, 82 ± 3%, $n = 8$; F2, 83 ± 5%, $n = 6$; $P > 0.05$, one-way ANOVA). The antihypertensive effects of F1 and F2 in SHR at a dose of 10 mg/kg are shown in **Figure 3B**. A significant decrease in SBP was observed for F1 1 h after administration (−11.4 ± 2.7 mmHg), whereas the slight decrease in SBP caused by F2 was not significant.

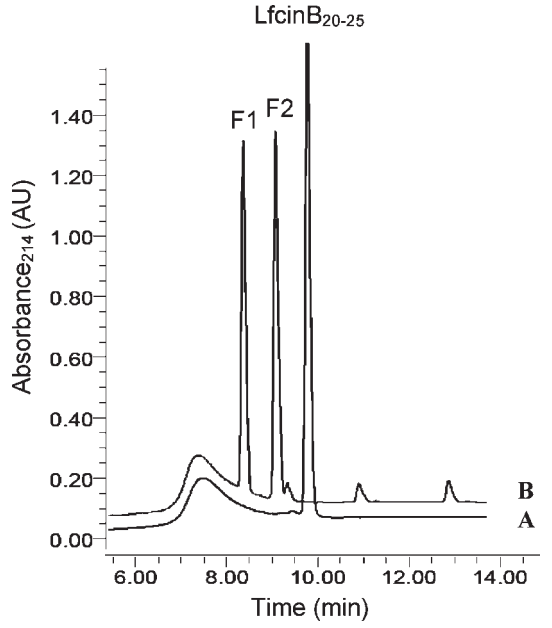

Figure 4. RP-HPLC chromatograms of $LfcinB_{20-25}$ before (A) and after (B) being submitted to simulated gastrointestinal digestion. $LfcinB_{22-23}$-F1 (WQ) and $LfcinB_{21-23}$-F2 (RWQ) were identified by RP-HPLC-MS/MS.

DISCUSSION

In a previous work, we showed the vasoactive effects of the antimicrobial LfcinB-derived peptide $LfcinB_{17-31}$ through ACE inhibition (*16*). In this study, we further characterized the antihypertensive activity of $LfcinB_{17-31}$ and of a set of LfcinB-derived peptides obtained from different N-terminal and C-terminal elongations of $LfcinB_{20-25}$, including in vitro ACE inhibitory capacity determinations, ex vivo functional assays using arterial segments, and in vivo studies in SHR.

LfcinB-derived peptides showed ACE inhibitory activity in the micromolar range (**Table 2**) with IC_{50} values of the same order

of magnitude as those reported for ACE inhibitory peptides derived from different milk proteins (*24*). Despite the pioneering studies about ACE inhibitory peptides derived from snake venom and their structural analogues which showed the importance of the C-terminal dipeptide hydrophobic sequences of ACE substrates and inhibitors (*25*−*27*), the effect of primary structure on potency is not fully understood. In our study, the peptides LfcinB$_{19-25}$, LfcinB$_{18-25}$, and LfcinB$_{17-25}$, derived from elongations at the N-terminal end of LfcinB$_{20-25}$, showed higher in vitro potency than the parental one. All of them have R as the C-terminal residue whose positive charge has been described to substantially contribute to ACE inhibitory potency in casein-derived peptides (*28*), although it does not fit with the rule proposed by Cheung et al. (*27*) about residues being preferred in penultimate (V, I, A, R, Y, and F) and ultimate positions (W, Y, F, P, I, A, L, and M) of ACE inhibitors and substrates. The different ACE inhibitory potency found between LfcinB$_{17-25}$ and LfcinB$_{17-24}$ reinforces the importance of the R residue at the C-terminal position, whereas the differences between LfcinB$_{17-31}$ and LfcinB$_{17-32}$ support the role of the C-terminal P residue in enhancing inhibition (*27*). Quantitative structure−activity modeling of ACE inhibitory peptides derived from milk proteins showed no relationship between N-terminal structure and inhibition activity, although it was suggested that in peptides longer than six amino acids in length, steric effects might be important (*4*). The results obtained with the three above-mentioned peptides confirm that N-terminal elongations may increase in vitro inhibitory potency, although it might not result in higher hypotensive activity in vivo (see below).

Ex vivo experiments using isolated rabbit carotid arteries add functional evidence for the ACE inhibitory effects of LfcinB-derived peptides in vascular tissue. Most of the peptides showed inhibitory effects on ACE-dependent, angiotensin I-induced contractions in the carotid artery (**Table 3**). Quite interestingly, LfcinB$_{19-25}$ with a high in vitro inhibitory potency (IC_{50} 2.3 ± 0.2 μM) did not show any effect on ACE-dependent vasoconstriction in contrast to LfcinB$_{20-25}$, LfcinB$_{17-31}$, and LfcinB$_{17-22}$ that had in vitro IC_{50} values approximately 10-fold higher. These results point out the lack of correlation between in vitro and ex vivo results in spite of using angiotensin I as the ACE substrate in both experiments. In our previous study, other LfcinB-related peptides with in vitro ACE inhibitory activity did not show ex vivo effects (*16*). In the context of the peptides studied, the addition of a C residue to the N-terminal end of LfcinB$_{20-25}$ provoked the loss of LfcinB$_{19-25}$ inhibitory activity, although the successive addition of K (LfcinB$_{18-25}$) and F (LfcinB$_{17-25}$) restored the activity. In contrast to that found in in vitro studies, the addition of a P residue to the C-terminal end of LfcinB$_{17-31}$ did not enhance the inhibitory effect on ACE-dependent vasoconstriction. These results indicate the complex structure−activity relationships in peptides longer than three residues and confirm previous data on how minor amino acid changes affect bioactive properties (*29*).

In the in vivo experiments, only LfcinB$_{20-25}$ showed a moderate hypotensive effect (−16.7 ± 3.2 mmHg) in SHR, that is, 7.7% reduction from baseline SBP. Numerous rat studies have been performed to determine the hypotensive effect of food-protein-derived ACE inhibitors, and in general, SBP reductions ranging from −0.6 to −80.0 mmHg have been reported (*2*). The low bioavailability of in vitro ACE inhibitory peptides following oral administration may be the cause for the lack of hypotensive effect in vivo. Peptides need to be absorbed intact and efficiently and be resistant to degradation by serum peptidases in order to reach the target organ. On the contrary, peptide degradation or fragmentation during gastrointestinal digestion or vascular circulation may result in more potent ACE inhibitory activities (*30*). When subjected to a simulated gastrointestinal digestion, all of the peptides tested in this study were hydrolyzed to different degrees, and remarkably, LfcinB$_{20-25}$ was completely hydrolyzed, strongly suggesting that its in vivo hypotensive effect may be due to derivative fragments. In fact, one of the fragments identified (F1: WQ) showed a moderate hypotensive effect (−11.4 ± 2.7 mmHg) in SHR, that is 5.8% reduction from baseline SBP, lower than that caused by LfcinB$_{20-25}$. The tripeptide RWQ (F2) also showed a slight hypotensive effect in SHR lower than that of the nested dipeptide, and under our experimental conditions, it was not statistically significant. It is worthwhile to note that protocols for in vitro gastrointestinal digestion may not completely reflect the physiological process and that intact LfcinB$_{20-25}$ may be responsible for the in vivo effect. The possibility of a synergistic effect among LfcinB$_{20-25}$ and its fragments cannot be discarded either. In the simulated digestion model, intestinal transport and resistance to degradation by serum peptidases have not been addressed. Peptides can be absorbed intact through the intestine by different mechanisms but may also be susceptible, in addition to brush border peptidases, to intracellular peptidase activities (*31*), and it is also known that many peptides are degraded within seconds or minutes in the bloodstream (*32*). In some cases, the utility of simulated gastrointestinal digestion and serum incubation as a screening protocol for the assessment of the potential of an ACE inhibitory peptide to act in vivo has been shown (*22*), although in vivo processing of peptides deserves further investigation. Interestingly, there is evidence of the generation of lactoferrin-derived peptides containing the LfcinB region, as peptides tested in this study, in the human stomach (*33*). Also, two peptides derived from a different lactoferrin region [f(382−389) and f(442−447)] were identified in the human intestine 20 min after milk ingestion (*34*).

In contrast to that found for LfcinB$_{20-25}$, the hypotensive fragment WQ did not show any effect on ACE-dependent vasoconstriction. This result agreed with the in vitro value of IC_{50} (> 100 μM) determined for the LfcinB$_{20-25}$ digest, suggesting a mechanism of hypotensive action other than ACE inhibition. It has been described that the release of vasodilatory substances such as prostaglandin, NO, or CO could also contribute to the blood pressure-lowering effects of various ACE inhibitory peptides (*24*). Recently, argininosuccinate synthetase, an important player in the citrulline-NO cycle that represents a potential limiting step in NO synthesis, has been suggested as a functional target for a snake venom antihypertensive peptide (*35*). Moreover, the modulation of endothelin-1 and an opioid receptor mechanism may also play a role in the antihypertensive effects of the ACE inhibitory milk-derived peptides lactokinin (*36*) and α-lactorphin (*37*), respectively.

To the best of our knowledge, LfcinB$_{20-25}$ is the first lactoferrin-derived peptide described as having an antihypertensive effect after oral administration in SHR. Despite the in silico study of Vermeirssen et al. (*38*) suggesting the potential of LF as a source of ACE inhibitory peptides, only one inhibitory peptide isolated from a pepsin hydrolysate (LRPVAA) has been identified and its antihypertensive effect stated after intravenous injection in SHR (*15*). Thus, we have shown that LfcinB$_{20-25}$, as many milk protein-derived peptides, possesses multifunctional properties since it can exert both antimicrobial (*18*) and antihypertensive effects. Further in vivo studies are being conducted to clarify the mechanisms involved in the hypotensive effect of LfcinB$_{20-25}$ and its derived dipeptide F1.

ABBREVIATIONS USED

LfcinB, lactoferricin B; ACE, angiotensin I-converting enzyme; SHR, spontaneously hypertensive rat; LF, lactoferrin; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; RP-HPLC-MS/MS, reversed-phase high-performance liquid chromatography−tandem mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; SEM, standard error of the mean; SBP, systolic blood pressure.

ACKNOWLEDGMENT

We thank María C. Máñez for her technical assistance.

LITERATURE CITED

(1) Hong, F.; Ming, L.; Yi, S.; Zhanxia, L.; Yongquan, W.; Chi, L. The antihypertensive effect of peptides: a novel alternative to drugs? *Peptides* **2008**, *29*, 1062–1071.

(2) Murray, B. A.; FitzGerald, R. J. Angiotensin converting enzyme inhibitory peptides derived from food proteins: biochemistry, bioactivity and production. *Curr. Pharm. Des.* **2007**, *13*, 773–791.

(3) Campbell, D. J. The renin-angiotensin and the kallikrein-kinin systems. *Int. J. Biochem. Cell Biol.* **2003**, *35*, 784–791.

(4) Pripp, A. H.; Isaksson, T.; Stepaniak, L.; Sørhaug, T. Quantitative structure-activity relationship modelling of ACE-inhibitory peptides derived from milk proteins. *Eur. Food Res. Technol.* **2004**, *219*, 579–583.

(5) Wu, J.; Aluko, R. E.; Nakai, S. Structural requirements of angiotensin I-converting enzyme inhibitory peptides: quantitative structure-activity relationship modelling of peptides containing 4−10 amino acid residues. *QSAR Comb. Sci.* **2006**, *10*, 873–880.

(6) Wu, J.; Aluko, R. E.; Nakai, S. Structural requirements of angiotensin I-converting enzyme inhibitory peptides: quantitative structure-activity relationship study of di- and tripeptides. *J. Agric. Food Chem.* **2006**, *54*, 732–738.

(7) Foltz, M.; van Buren, L.; Klaffke, W.; Duchateau, G. S. M. J. E. Modeling of the relationship between dipeptide structure and dipeptide stability, permeability, and ACE inhibitory activity. *J. Food Sci.* **2009**, *74*, H243–H251.

(8) Vermeirssen, V.; van Camp, J.; Verstraete, W. Bioavailability of angiotensin I converting enzyme inhibitory peptides. *Br. J. Nutr.* **2004**, *92*, 357–366.

(9) López-Fandiño, R.; Otte, J.; van Camp, J. Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. *Int. Dairy J.* **2006**, *16*, 1277–1293.

(10) Haque, E.; Chand, R. Antihypertensive and antimicrobial bioactive peptides from milk proteins. *Eur. Food Res. Technol.* **2008**, *227*, 7–15.

(11) Otte, J.; Shalaby, S. M.; Zakora, M.; Pripp, A. H.; El-Shabrawy, S. H. Angiotensin-converting enzyme inhibitory activity of milk protein hydrolysates: effect of substrate, enzyme and time of hydrolysis. *Int. Dairy J.* **2007**, *17*, 488–503.

(12) Pihlanto-Leppälä, A. Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. *Trends Food Sci. Technol.* **2001**, *11*, 347–356.

(13) Wakabayashi, H.; Yamauchi, K.; Takase, M. Lactoferrin research, technology and applications. *Int. Dairy J.* **2006**, *16*, 1241–1251.

(14) Gifford, J. L.; Hunter, H. N.; Vogel, H. J. Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. *Cell. Mol. Life Sci.* **2005**, *62*, 2588–2598.

(15) Lee, N.-Y.; Cheng, J.-T.; Enomoto, T.; Nakamura, I. The antihypertensive activity of angiotensin-converting enzyme inhibitory peptide containing in bovine lactoferrin. *Chin. J. Physiol.* **2006**, *49*, 67–73.

(16) Centeno, J. M.; Burguete, M. C.; Castelló-Ruiz, M.; Enrique, M.; Vallés, S.; Salom, J. B.; Torregrosa, G.; Marcos, J. F.; Alborch, E.; Manzanares, P. Lactoferricin-related peptides with inhibitory effects on ACE-dependent vasoconstriction. *J. Agric. Food Chem.* **2006**, *54*, 5323–5329.

(17) Ruiz-Giménez, P.; Burguete, M. C.; Castelló-Ruiz, M.; Marcos, J. F.; Salom, J. B.; Vallés, S.; Torregrosa, G.; Alborch, E.; Manzanares, P. Bovine lactoferrin pepsin hydrolysate exerts inhibitory effect on ACE-dependent vasoconstriction. *Int. Dairy J.* **2007**, *17*, 1212–1215.

(18) Tomita, M.; Takase, M.; Bellamy, W.; Shimamura, S. A review: the active peptide of lactoferrin. *Acta Paediatr. Jpn.* **1994**, *36*, 585–591.

(19) Wu, J.; Aluko, R. E.; Muir, A. D. Improved method for direct high-performance liquid chromatography assay of angiotensin-converting enzyme-catalyzed reactions. *J. Chromatogr., A* **2002**, *950*, 125–130.

(20) Obernier, J. A.; Baldwin, R. L. Establishing an appropriate period of acclimatization following transportation of laboratory animals. *ILAR J.* **2006**, *47*, 364–369.

(21) Ibrahim, J.; Berk, B. C.; Hughes, A. D. Comparison of simultaneous measurements of blood pressure by tail-cuff and carotid arterial methods in conscious spontaneously hypertensive and Wistar-Kyoto rats. *Clin. Exp. Hypertens.* **2006**, *28*, 57–72.

(22) Walsh, D. J.; Bernard, H.; Murray, B. A.; MacDonald, J.; Pentzien, A.-K.; Wright, G. A.; Wal, J.-M.; Struthers, A. D.; Meisel, H.; FitzGerald, R. J. *In vitro* generation and stability of the lactokinin β-lactoglobulin fragment (142−148). *J. Dairy Sci.* **2004**, *87*, 3845–3857.

(23) Vermeirssen, V.; Augustijns, P.; Morel, N.; van Camp, J.; Opsomer, A.; Verstraete, W. *In vitro* intestinal transport and antihypertensive activity of ACE inhibitory pea and whey digests. *Int. J. Food Sci. Nutr.* **2005**, *56*, 415–430.

(24) De Leo, F.; Panarese, S.; Gallerani, R.; Ceci, L. R. Angiotensin converting enzyme (ACE) inhibitory peptides: production and implementation of functional food. *Curr. Pharm. Des.* **2009**, *15*, 3622–3643.

(25) Cushman, D. W.; Pluscec, J.; Williams, N. J.; Weaver, E. R.; Sabo, E. F.; Kocy, O.; Cheung, H. S.; Ondetti, M. A. Inhibition of angiotensin-converting enzyme by analogues of peptides from *Bothrops jararaca* venom. *Experientia* **1973**, *29*, 1032–1035.

(26) Cheung, H. S.; Cushman, D. W. Inhibition of homogeneous angiotensin I-converting enzyme of rabbit lung by synthetic venom peptides of *Bothrops jararaca*. *Biochim. Biophys. Acta* **1973**, *293*, 451–463.

(27) Cheung, H. S.; Wang, F. L.; Ondetti, M. A.; Sabo, E. F.; Cushman, D. W. Binding of peptide substrates and inhibitors of angiotensin-converting enzyme: importance of the COOH-terminal dipeptide sequence. *J. Biol. Chem.* **1980**, *255*, 401–407.

(28) FitzGerald, R. J.; Meisel, H. Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *Br. J. Nutr.* **2000**, *84*, S33–S37.

(29) López-García, B.; Pérez-Payá, E.; Marcos, J. F. Identification of novel hexapeptides bioactive against phytopathogenic fungi through screening of a synthetic peptide combinatorial library. *Appl. Environ. Microbiol.* **2002**, *68*, 2453–2460.

(30) FitzGerald, R. J.; Murray, B. A.; Walsh, D. J. The emerging role of dairy proteins and bioactive peptides in nutrition and health: hypotensive peptides from milk proteins. *J. Nutr.* **2004**, *134*, 980–988.

(31) Webb, K. E., Jr Intestinal absorption of protein hydrolysis products: a review. *J. Anim. Sci.* **1990**, *68*, 3011–3022.

(32) Fuglsang, A.; Nilsson, D.; Nyborg, N. C. B. Characterization of new milk-derived inhibitors of angiotensin converting enzyme *in vitro and in vivo*. *J. Enzyme Inhib. Med. Chem.* **2003**, *18*, 407–412.

(33) Kuwata, H.; Yip, T.-T.; Tomita, M.; Hutchens, T. W. Direct evidence of the generation in human stomach of an antimicrobial peptide domain (lactoferricin) from ingested lactoferrin. *Biochim. Biophys. Acta* **1998**, *1429*, 129–141.

(34) Chabance, B.; Marteau, P.; Rambaud, J. C.; Migliore-Samour, D.; Boynard, M.; Perrotin, P.; Guillet, R.; Jollès, P.; Fiat, A. M. Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie* **1998**, *80*, 155–165.

(35) Guerreiro, J. R.; Lameu, C.; Oliveira, E. F.; Klitzke, C. F.; Melo, R. L.; Linares, E.; Augusto, O.; Fox, J. W.; Lebrun, I.; Serrano, S. M. T.; Camargo, A. C. M. Argininosuccinate synthetase is a functional target for a snake venom anti-hypertensive peptide: role in arginine and nitric oxide production. *J. Biol. Chem.* **2009**, *284*, 20022–20033.

(36) Maes, W.; van Camp, J.; Vermeirssen, V.; Hemeryck, M.; Ketelslegers, J. M.; Schrezenmeir, J.; van Oostveldt, P.; Huyghebaert, A. Influence of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg (ALPMHIR) on the release of endothelin-1 by endothelial cells. *Reg. Peptides* **2004**, *118*, 105–109.
(37) Nurminen, M.-L.; Sipola, M.; Kaarto, H.; Pihlanto-Leppälä, A.; Piilola, K.; Korpela, R.; Tossavainen, O.; Korhonen, H.; Vapaatalo, H. α-Lactorphin lowers blood pressure measured by radiotelemetry in normotensive and in spontaneously hypertensive rats. *Life Sci.* **2000**, *66*, 1535–1543.
(38) Vermeirssen, V.; van der Bent, A.; van Camp, J.; van Amerongen, A.; Verstraete, W. A quantitative in silico analysis calculates the angiotensin I converting enzyme (ACE) inhibitory activity in pea and whey protein digests. *Biochimie* **2004**, *86*, 231–239.

Received for review March 8, 2010. Revised manuscript received April 22, 2010. Accepted April 26, 2010. This work was supported by grants AGL2007-64672/ALI from 'Ministerio de Educación y Ciencia-FEDER', Consolider Ingenio 2010, Fun-C-Food, CSD2007-00063, and RETICS-RD06/0026/0006 from 'Instituto de Salud Carlos III'. P.R.-G. is a recipient of a fellowship from 'Ministerio de Educación y Ciencia' (BES-2005-10382).