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Lactoferricin-Related Peptides with Inhibitory Effects on ACE-Dependent Vasoconstriction

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A selection of lactoferricin B (LfcinB)-related peptides with an angiotensin I-converting enzyme (ACE) inhibitory effect have been examined using in vitro and ex vivo functional assays. Peptides that were analyzed included a set of sequence-related antimicrobial hexapeptides previously reported and two representative LfcinB-derived peptides. In vitro assays using hippuryl-L-histidyl-L-leucine (HHL) and angiotensin I as substrates allowed us to select two hexapeptides, PACEI32 (Ac-RKWHFW-NH_2) and PACEI34 (Ac-RKWLFW-NH_2), and also a LfcinB-derived peptide, $LfcinB_{17-31}$ (Ac-FKCRRWQWRMKKLGA-NH_2). Ex vivo functional assays using rabbit carotid arterial segments showed PACEI32 (both D- and L-enantiomers) and $LfcinB_{17-31}$ have inhibitory effects on ACE-dependent angiotensin I-induced contraction. None of the peptides exhibited in vitro ACE inhibitory activity using bradykinin as the substrate. In conclusion, three bioactive lactoferricin-related peptides exhibit inhibitory effects on both ACE activity and ACE-dependent vasoconstriction with potential to modulate hypertension that deserves further investigation.

KEYWORDS: Lactoferricin B-related peptides; ACE inhibition; ex vivo functional assay; ACE-dependent vasoconstriction

INTRODUCTION

Angiotensin I-converting enzyme (ACE, dipeptidyl-carboxypeptidase, EC 3.4.15.1) is an important drug target in the treatment of cardiovascular diseases, especially hypertension. ACE plays an important role in the renin−angiotensin system which regulates both arterial blood pressure and the salt−water balance (*1*). ACE catalyzes the cleavage of the C-terminal dipeptide from angiotensin I (DRVYIHPFHL) to produce angiotensin II (DRVYIHPF), an octapeptide which has a potent vasoconstrictor effect. The in vivo effect of vasoactive nonapeptide bradykinin (RPPGFSPFR) is hypotensive vasodilatation, and it may have cardioprotective effects as well (*2*, *3*) and be inactivated by ACE through two successive cleavages to pentapeptide BK1-5 (RPPGF) (*4*). Since the first discovery of ACE inhibitory peptides from snake venom (*5*) and the successful development of captopril as the first orally active peptide-based ACE inhibitor (*6*), a variety of potent inhibitors have been discovered or synthesized. However, synthetic available ACE inhibitors have certain side effects such as cough, taste disturbances, and skin rashes (*7*). Therefore, interest in identifying natural sources of peptide ACE inhibitors as a healthier alternative to the inhibitory drugs has grown.

In this context, biological functions of food constituents have been elucidated. Among them, peptides with different biological activities, including ACE inhibitors, have been described. Research on ACE inhibitory peptides has mainly focused on milk proteins (*8*), but vegetable and other animal proteins have been also studied (*9*). The identification of bioactive peptides mainly includes isolation from food protein hydrolysates (*10*−*12*), although some ACE inhibitory peptides were directly isolated from food without in vitro proteolysis (*13*, *14*). Some

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

peptide	sequence
P19D	Ac-rktwfw-NH_2
P20D	Ac-rktpfw-NH_2
P26D	Ac-rkkwfw-NH_2
P32D (PACEI32D)	Ac-rkwhfw-NH_2
P34D (PACEI34D)	Ac-rkwlfw-NH_2
P36D	Ac-rkwrfw-NH_2
P37D	Ac-rkkpfw-NH_2
PACEI32L	Ac-RKWHFW-NH_2
PACEI34L	Ac-RKWLFW-NH_2
$LfcinB_{20-25}$	Ac-RRWQWR-NH_2
$LfcinB_{17-31}$	Ac-FKCRRWQWRMKKLGA-NH_2

[a] The L-amino acids are shown in uppercase letters, and the D-amino acids are shown in lowercase letters. All the peptides were acetylated at the N-terminus (Ac) and amidated at the C-terminus (NH_2).

strategies were based on searching within natural proteins sequence similarities to ACE inhibitory peptides and the synthesis of peptides thereof (*15*, *16*). Some of the identified ACE inhibitory peptides exhibit significant antihypertensive effects, although their inhibitory potencies on ACE activity do not always correlate with their antihypertensive activities (*9*, *17*).

Some bioactive peptides possess multiple functions simultaneously, i.e., antioxidant and ACE inhibitory activities (*18*). Previously, we have used an unbiased combinatorial approach to identify a series of sequence-related D-hexapeptides, with specific antimicrobial activity against phytopathogenic fungi (*19*). All of them share amino acid sequences commonly found in ACE inhibitory peptides, in particular, a C-terminal FW dipeptide motif (*20*, *21*). We have also observed that the sequences of these peptides are strongly similar with that of the antibacterial core of lactoferrin B, a well-known milk protein (*22*).

Lactoferrin (LF), a major constituent of mammal milk, is a multifunctional iron glycoprotein which is known to exhibit a diverse range of biological activities, including antimicrobial, antiviral, and antioxidant activities, as well as immunomodulation (*23*). Lactoferricin B (LfcinB) is a 25-residue antimicrobial peptide derived from pepsin-digested bovine LF that shares some biological activities with LF and possesses antimicrobial activity (*24*). LfcinB-related peptides are also known to cause inhibition of platelet aggregation, to inhibit the growth of mammalian cell lines, especially tumor cells, and to bind to various biological compounds (*25*, *26*). Although milk proteins, both caseins and whey proteins, are a rich source of ACE inhibitory peptides (*8*), there is no information about such biological activity in Lfcin-related peptides.

In this study, we investigated the ACE inhibitory effects of LfcinB-related peptides, including a set of hexapeptides and $LfcinB_{20-25}$ and $LfcinB_{17-31}$, two representative LfcinB-derived peptides (*27*, *28*). Peptides exhibiting high ACE inhibitory activity in vitro were examined for their ACE inhibitory effects in ex vivo functional assays.

MATERIALS AND METHODS

Materials. Hippuryl-L-histidyl-L-leucine (HHL), ACE (from porcine kidney), bradykinin fragment 1-5, and captopril were purchased from Sigma Chemical Co. (St. Louis, MO). Angiotensin I, angiotensin II, and bradykinin were provided by Calbiochem Co. (La Jolla, CA).

Peptide Synthesis. Peptides (**Table 1**) were purchased at >90% purity from GenScript Corp. (Piscataway, NJ) wherein they were synthesized by solid-phase methods using *N*-(9-fluorenyl)methoxycarbonyl (Fmoc) chemistry. All the peptides were acetylated at the N-terminus and amidated at the C-terminus. Stock solutions of each peptide were prepared at a concentration of 1 mM in 5 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 7) and stored at −20 °C. Peptide concentrations were determined by measuring the absorbance at 280 nm ($\epsilon^{280} = 5600\ M^{-1}\ cm^{-1}$ for a W residue).

In Vitro Assays of ACE Inhibitory Activity. Peptides were assayed in vitro for their capacity to inhibit the ACE activity using HHL, angiotensin I, and bradykinin as substrates and quantifying the reaction products hippuric acid, angiotensin II, and bradykinin fragment 1-5, respectively, by RP-HPLC (*29*).

Peptide solutions (200 μM) in 10 mM MOPS buffer (pH 7, up to 125 μL) and 75 μL of a 20 munits/mL ACE solution in 200 mM Tris-HCl buffer (pH 8.3) containing 600 mM NaCl and 10 μM $ZnCl_2$ were preincubated at 37 °C for 15 min, and the mixture was incubated with 50 μL of 25 mM HHL in the same buffer for 30 min at the same temperature. The reaction was terminated by the addition of 25 μL of 6 M HCl. When using natural substrates for ACE activity, HHL was substituted with 50 μL of 0.31 mM angiotensin I or 20 μL of 0.94 mM bradykinin.

HPLC was performed on a Waters system (Waters Corp., Milford, MA) equipped with a 1525 Binary HPLC pump, a 2487 dual λ absorbance detector, and a 717 plus autosampler. Reaction mixtures (200 μL) were analyzed on a Symmetry C_{18} column (4.6 mm × 150 mm, 5 μm, Waters) kept at 40 °C. The column was developed at a flow rate of 1 mL/min. Hippuric acid was eluted using an acetonitrile gradient containing 0.05% TFA (from 5 to 60% in 10 min and maintained for 2 min at 60%) and detected at 228 nm. Angiotensin II and bradykinin fragment 1-5 were eluted using an acetonitrile gradient containing 0.1% TFA (from 20 to 40% over the course of 10 min) and detected at 214 nm.

Data are expressed as the percentage of ACE residual activity with respect to a control without peptide (100%) and are means ± SD. Tukey's honestly significant difference procedure (HSD) was used for mean separation (StatGraphics Plus 5.1, StatPoint, Herndon, VA).

The IC_{50} value of a peptide was defined as the concentration required to inhibit 50% of the ACE activity, and the value for each experiment was estimated by nonlinear regression of the experimental data to a four-parameter logistic curve using SigmaPlot version 8.02 (SPSS Inc., Chicago, IL).

Animals and Arteries. Experiments were conducted in compliance with the Spanish legislation on Protection of Animals used for Experimental and other Scientific Purposes and in accordance with the Directives of the European Community on this subject. Thirty male New Zealand white rabbits (Technology Transferring Center, Polytechnic University of Valencia, Valencia, Spain), weighing 2.5−3 kg, were killed by injection of 25 mg of sodium thiopental per kilogram (Tiobarbital, B Braun Medical, Jaén, Spain) and 1.5 mL of a 10 mM KCl solution through the ear vein. A midline throat incision provided access to the common carotid artery, which was dissected free and cut in four 4 mm long segments.

Ex Vivo Functional Assay of ACE Inhibitory Effect. For isometric tension recording, the arterial segments were mounted in an organ bath by using tungsten wires (207 μm in diameter). Two pins were introduced through the arterial lumen: one pin fixed to a stationary support and the other connected to a strain gauge (Universal Transducing Cell UC3, Gould Statham, Oxnard, CA). Isometric tension was conveniently amplified (OCTAL Bridge, ADInstruments, Castle Hill, Australia), digitized (PowerLab/8SP, ADInstruments), recorded, and stored in an IBM personal computer by means of the appropriate software (Chart 5, ADInstruments) for later analysis. Each organ bath contained 5 mL of 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) at 37 °C and bubbled with a 95% O_2/5% CO_2 mixture to give a pH of 7.3−7.4. A previously determined optimal resting tension of 2 g was applied to the carotid arterial segments, and they were allowed to equilibrate for 30−60 min before the experiments were started.

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 1 g were discarded. Then, every arterial segment was challenged with a single concentration (1 μM) of angiotensin I. After being washed

Table 2. ACE Inhibitory Activity of D-Amino Acid Peptides

peptide[a]	ACE residual activity (%)[b]
P19D	126 ± 22 (3) (a)
P20D	133 ± 19 (3) (a)
P26D	100 ± 6 (4) (ab)
P32D	52 ± 13 (4) (c)
P34D	68 ± 12 (4) (c)
P36D	97 ± 4 (7) (ab)
P37D	76 ± 6 (4) (bc)

[a] Final concentration in the assay of 20 μM. [b] Data are expressed as the percentage of ACE residual activity with respect to a control without peptide (100%) using HHL as substrate and are the means ± SD from *n* experiments. Data with the same letter do not differ at the 95% level of confidence (Tukey's HSD procedure).

Table 3. ACE Inhibitory Activity of L-Amino Acid Peptides

	ACE residual activity (%)[b]	
peptide[a]	HHL	angiotensin I
PACEI32L	58 ± 15 (5) (b)	45 ± 11 (5) (bc)
PACEI34L	74 ± 8 (6) (a)	29 ± 9 (4) (c)
$LfcinB_{20-25}$	71 ± 8 (6) (ab)	66 ± 6 (4) (a)
$LfcinB_{17-31}$	62 ± 6 (4) (ab)	59 ± 11 (7) (ab)

[a] Final concentration in the assay of 20 μM. [b] Data are expressed as the percentage of ACE residual activity with respect to a control without peptide (100%) and are the means ± SD from *n* experiments. Data with the same letter do not differ at the 95% level of confidence (Tukey's HSD procedure). All values differed from the control with no peptide added at the 99% level of confidence.

out, each arterial segment was subjected to one of the following protocols: a second challenge to angiotensin I to check for response reproducibility, preincubation with captopril (from 1 nM to 1 μM) and a second challenge to angiotensin I to check for the ACE dependence of the response to angiotensin I, or preincubation with one of the peptides (20 μM) and a second challenge to angiotensin I to check for their effect on angiotensin I-induced contraction. Additionally, in some arterial segments, negative controls for ACE dependence were obtained by challenging twice with angiotensin II (1 μM), with captopril (from 0.1 μM to 1 μM) preincubation between the challenges.

All data are expressed as means ± SEM from *n* arterial segments obtained from *n* different rabbits. The second contractile responses to angiotensins I and II are expressed as a percentage of the first contraction in the same arterial segment. One-way ANOVA followed by a Dunnett multiple comparison test was used to compare the control and experimental groups (GraphPad Instat 3.06, GraphPad Software Inc., San Diego, CA). $P < 0.05$ was considered significant.

RESULTS

In Vitro ACE Inhibitory Activity of D-Amino Acid Hexapeptides. Measurements of ACE inhibitory activity were carried out with HHL as the substrate, a widely used synthetic analogue of the C-terminal dipeptide of angiotensin I (*30*). **Table 2** shows the ACE residual activity of seven D-amino acid hexapeptides when tested at a concentration of 20 μM. All the peptides share W and F residues at the C-terminus and R and K residues at the N-terminus. Among these peptides, the highest inhibitory activities with HHL as the substrate were seen for P32D and P34D, with H and L, respectively, at position 4, indicating that these are preferred over R at this position (P36D) in the context of this hexapeptide sequence. P32D, P34D, and P37D were significantly different from the no peptide control (ACE activity of 100%) at the 95% confidence level (Student's *t* test). Therefore, P37D exhibited an intermediate effect since its calculated mean value was not different from those of nonactive peptides P26D and P36D (Tukey HSD test, **Table 2**). On the other hand, peptides P19D, P20D, P26D, and P36D did not exhibit significant differences among them or with the control. This suggests that P or W at position 4 is not suitable for ACE inhibition in the sequence framework defined by this peptide set. The amino acid residue at position 3 (K instead of T) is the only difference between P37D and P20D and could explain the absence of an inhibitory effect of P20D.

Peptides P32D and P34D were selected for further studies and renamed as PACEI (from peptide and ACE inhibitor).

In Vitro ACE Inhibitory Activity of L-Amino Acid Peptides. The L-amino acid counterparts of PACEI32D and PACEI34D were tested for their capacity for ACE inhibition, at 20 μM, using as substrates HHL and angiotensin I, one of the physiologically active substrates of ACE (**Table 3**). The two L-peptides derived from LfcinB, $LfcinB_{20-25}$ (6-mer) and $LfcinB_{17-31}$ (15-mer), were also included in the study at this point.

When using HHL, PACEI32L and PACEI34L exhibited ACE residual activities of 58 ± 15 and 74 ± 8%, respectively, in accordance with that found for their D-enantiomers (**Table 2**). Overall, the higher ACE inhibitory activity of these two peptides was found when using angiotensin I as substrate, showing PACEI32L and PACEI34L ACE residual activities of 45 ± 11 and 29 ± 9%, respectively. In fact, the in vitro activities when using the two alternative substrates were different for PACEI32L and PACEI34L with *P* values of 0.15731 and <0.0001, respectively (Student's *t* test), and therefore, the effect of changing the substrate was more pronounced in the case of the latter peptide.

In contrast, the LfcinB-derived peptides $LfcinB_{20-25}$ and $LfcinB_{17-31}$ exhibited similar ACE residual activities on both HHL and angiotensin I that were not statistically significant ($P > 0.5$).

Further experiments were carried out to determine the IC_{50} values for PACEI32L, PACEI34L, and $LfcinB_{17-31}$ with the natural substrate, angiotensin I. Since the sequence of $LfcinB_{20-25}$ is included in that of $LfcinB_{17-31}$ and could be released by the action in vivo of gastrointestinal proteases, we decided to select the 15-residue peptide for further studies. Results are shown in **Figure 1**. As can be seen, PACEI32L and PACEI34L exhibited IC_{50} values of 10.7 ± 4.1 and 8.1 ± 2.5 μM, whereas $LfcinB_{17-31}$ exhibited a higher IC_{50} value (25.5 ± 4.5 μM), in agreement with the inhibitory activities determined at a peptide concentration of 20 μM (**Table 3**).

Because in vivo ACE inhibition potentiates the hypotensive effects of bradykinin, we performed further experiments to determine the ACE inhibitory capacity of PACEI32, PACEI34, and $LfcinB_{17-31}$ using bradykinin as the substrate. None of the peptides tested at a concentration of 20 μM exhibited inhibitory effects (results not shown).

Ex Vivo Inhibitory Effects on ACE-Dependent Vasoconstriction. Challenge of rabbit carotid arterial segments with a depolarizing solution (50 mM KCl) induced phasic contraction followed by active tone maintained until KCl wash-out (**Figure 2A**). Average active tone amounted to 2749 ± 124 mg ($n = 120$). Angiotensin I (1 μM) induced phasic, transient contractions that were almost completely reproducible in two consecutive challenges to the same arterial segment (**Figure 2A**). Preincubation with the ACE inhibitor captopril (1 μM) completely abolished angiotensin I-induced contraction. This inhibition was reversible after captopril wash-out (**Figure 2B**, top). In contrast, angiotensin II-induced contraction was not significantly affected by captopril incubation (**Figure 2B**, bottom). **Table 4** summarizes concentration-dependent inhibitory effects

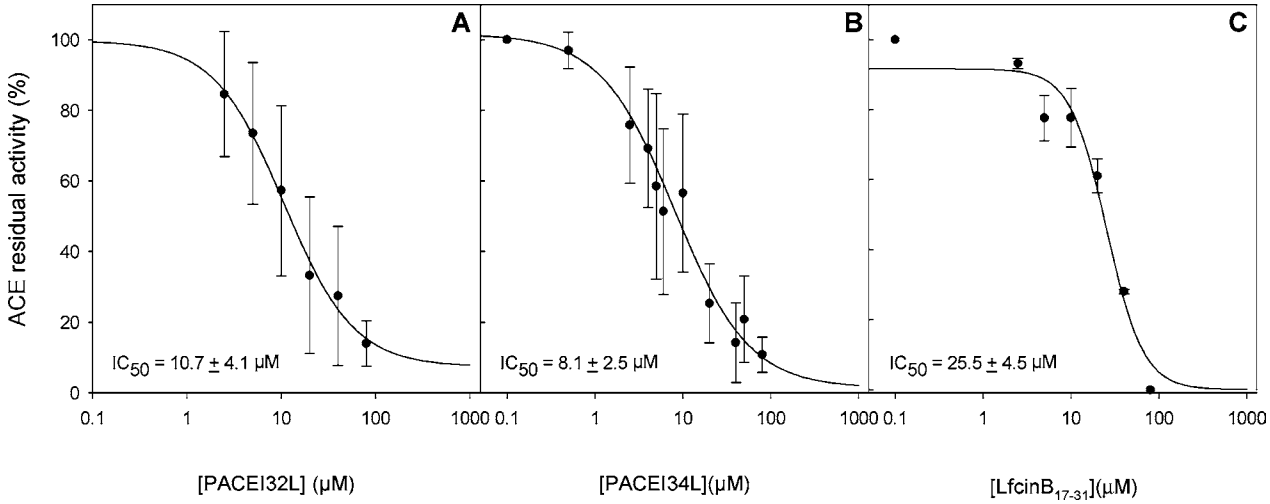

Figure 1. Effect of the concentration of PACEI32L (**A**), PACEI34L (**B**), and $LfcinB_{17-31}$ (**C**) on ACE residual activity. IC_{50} values are the means ± SD of at least three independent experiments.

of captopril on angiotensin I-induced contraction, the reversion after captopril wash-out, and the lack of an effect of the ACE inhibitor on angiotensin II-induced contraction.

The five peptides assayed for their ACE inhibitory capability exhibited different inhibitory effects on angiotensin I-induced contractions. As shown in **Table 5**, PACEI32D, PACEI32L, and $LfcinB_{17-31}$ (20 μM) preincubation induced significant inhibitions when compared to the control. By contrast, although contractions to angiotensin I in arterial segments preincubated with PACEI34D or PACEI34L (20 μM) were weaker than the control response, the reduction did not reach significance. **Figure 2C** shows the inhibitory effect of PACEI32L and $LfcinB_{17-31}$ on angiotensin I-induced contraction.

DISCUSSION

ACE inhibitors are now widely used for the treatment of hypertension and heart failure. Although originally it was thought that their beneficial effects were mainly due to the blockade of angiotensin II generation, recent evidence suggests that bradykinin accumulation may be equally important (*31*). Although there have been many studies on the ACE inhibitory activities of peptides, the relationship between the structure and ACE inhibitory activity of peptides has not yet been fully established and indicates the complexity of the inhibitory mechanism (*21*). It seems that binding to ACE is strongly influenced by the C-terminal tripeptide that can interact with subsites S_1, S_1', and S_2' at the active site of ACE (*32*).

In this study, we determined the in vitro ACE inhibitory capacity of LfcinB-related peptides using HHL and angiotensin I as substrates and examined this capacity in ex vivo functional assays using arterial segments. Although many studies have been performed on antihypertensive peptides derived from milk proteins (mainly caseins) (*8*, *9*) and distinct biological activities have been described for LfcinB-related peptides (*24*−*26*), there is a lack of information about the possible ACE inhibitory activities of these peptides.

The COOH-terminal dipeptide sequences of some peptides used in this study (**Table 1**) fulfill the rule proposed by Cheung et al. (*20*) 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. From the peptides that were studied, only three, PACEI32D, PACEI34D, and P37D, exerted a significant ACE inhibitory action. From our data, we conclude that residues at the third C-terminal position influence the inhibitory effect since C-terminal tripeptides HFW and LFW are preferred over RFW (P36D). Also, in the context of these peptide sequences, P and W at the third C-terminal position do have equal effects since there were no significant differences in ACE residual activity between P19D and P20D and between P26D and P37D (**Table 2**). The different ACE inhibitory pattern found between P37D and P20D can be explained only by the T residue in position 3 (P20D) instead of K (P37D), suggesting also the importance of the fourth C-terminal position, in agreement with the study of Gómez-Ruiz et al. (*33*) in which the substitution of a hydrophobic residue (V) with a dicarboxylic amino acid (G) at the fourth C-terminal position negatively affected ACE inhibitory activity. These results indicate the complex structure−activity relationship in peptides longer than three residues.

Our results showed that the difference in ACE inhibitory activity in relation to the substrate used in the in vitro determinations is dependent on peptide sequence since this effect was observed for PACEI32L and PACEI34L, but not for the two LfcinB-derived peptides despite their similar net charges and amino acid properties. This finding points to the suitability of using both types of substrate for ACE inhibitory activity determinations in future studies. In addition, it also suggests different inhibitory mechanisms for PACEIs and LfcinB-derived peptides. HHL mimics the dipeptide released from angiotensin I; hence, the LfcinB-derived peptides would interfere with recognition of this N-terminal motif in the natural substrate. However, PACEIs, which are much more inhibitory when angiotensin I is used rather than HHL, would have other effects on ACE in addition to this interference. In this context, it is of note that PACEI32L and PACEI34L were partially degraded in vitro upon preincubation with ACE (around 15% of the peptide was hydrolyzed), whereas $LfcinB_{17-31}$ was not (data not shown). Nevertheless, the inhibitory effect of PACEIs does not seem to be due to the action of putative ACE hydrolysis products since the D- and L-amino acid peptides exhibit very similar activities on HHL (compare data in **Tables 2** and **3**) and in ex vivo functional assays (**Table 5**), and D-peptides are not expected to be subjected to proteolytic degradation.

We have found a new application for the antimicrobial LfcinB-derived peptides $LfcinB_{20-25}$ and $LfcinB_{17-31}$ in the control of hypertension as ACE inhibitors. In our study, these peptides were synthesized with L-stereoisomers and were found to exhibit an ACE inhibitory activity of ∼40% at a concentration

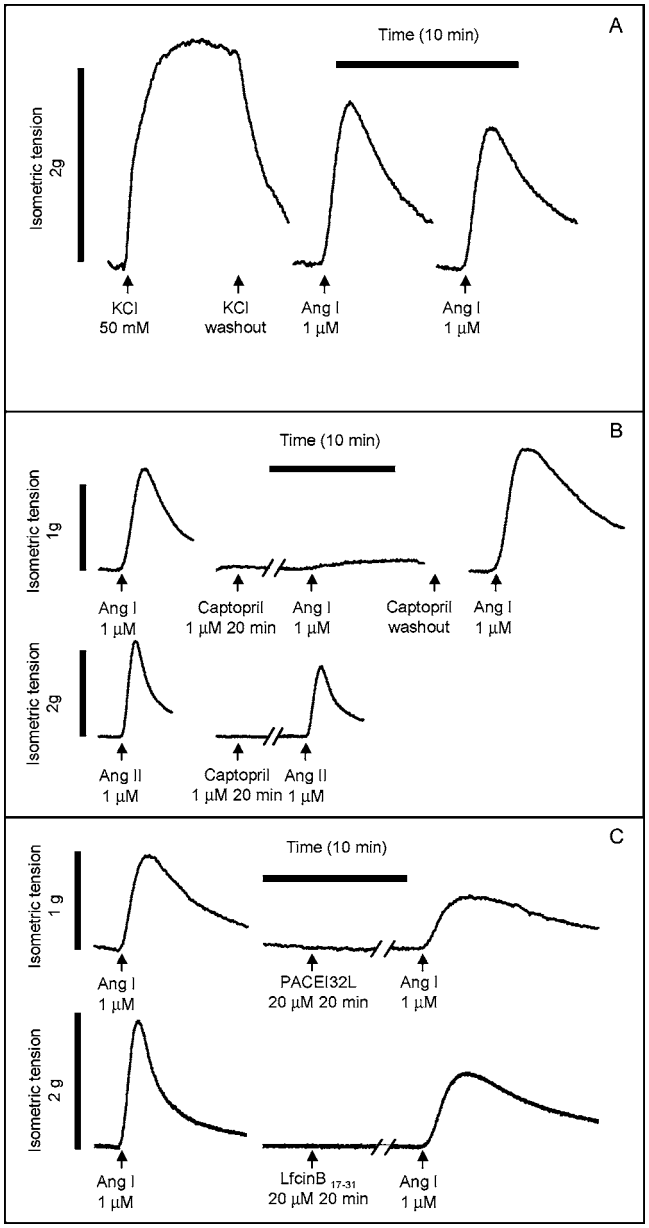

Figure 2. Contraction of carotid artery segments. (**A**) Effect of KCl to check for tissue viability and reproducible vasoconstriction to angiotensin I. (**B**) Effects of captopril on angiotensin I-induced (top) and angiotensin II-induced (bottom) contraction. (**C**) Effects of PACEI32L and $LfcinB_{17-31}$ on angiotensin I-induced contraction.

of 20 μM on both substrates, HHL and angiotensin I. There was no significant difference in ACE inhibitory activity between $LfcinB_{20-25}$ and $LfcinB_{17-31}$, suggesting that the elongation of $LfcinB_{20-25}$ at its C-terminus has no effect on ACE inhibition. Both derive from LfcinB, a 25-residue antibacterial peptide isolated after cleavage of bovine LF, and $LfcinB_{20-25}$ has been characterized as being its active core (*34*).

The highest inhibitory potency was found for peptides PACEI34L and PACEI32L followed by $LfcinB_{17-31}$ as shown by their IC_{50} values: 8.1 ± 2.5 μM (PACEI34L), 10.7 ± 4.1 μM (PACEI32L), and 25.5 ± 4.5 μM ($LfcinB_{17-31}$). These IC_{50} values are on the same order of magnitude as those reported for ACE inhibitory peptides derived from different food proteins (*9*). Nevertheless, these IC_{50} values are still far from the IC_{50} (0.022 μM) of the synthetic ACE inhibitor captopril (*6*). However, it has been reported that some ACE inhibitors possess better in vivo properties than the efficacy levels extrapolated from in vitro effects (*17*) probably due to the higher affinity of these peptides for tissue and slower elimination of them than of a drug compound such as captopril. However, it is not the objective of this study to replace ACE inhibitory drugs but to develop functional ingredients that integrated in food would provide a preventive more than curative treatment for hypertension without possible side effects.

Table 4. Concentration-Dependent Effects of Captopril on Angiotensin I- and Angiotensin II-Induced Contractions

	contraction peak (%)[a]	
	angiotensin I	angiotensin II
control	86 ± 3 (27)	58 ± 2 (9)
captopril, 1 nM	53 ± 3 (6)[b]	na[c]
captopril, 10 nM	12 ± 5 (6)[b]	na[c]
captopril, 0.1 μM	2 ± 2 (4)[b]	68 ± 11 (4)
captopril, 1 μM	0 (8)[b]	79 ± 1 (2)
captopril wash-out	120 ± 13 (5)[b]	na[c]

[a] Second contraction response to angiotensin I and II expressed as a percentage of the first contraction in the same arterial segment. Data are expressed as means ± SEM from *n* arterial segments. [b] Significantly different from the control ($P < 0.01$). [c] Not assayed.

Table 5. Effects of Studied Peptides on Angiotensin I-Induced Contractions

peptide[a]	contraction peak (%)[b]
control	86 ± 3 (27)
PACEI32D	65 ± 7 (11)[c]
PACEI32L	55 ± 4 (12)[c]
PACEI34D	73 ± 4 (6)
PACEI34L	67 ± 2 (6)
$LfcinB_{17-31}$	68 ± 6 (10)[d]
captopril	12 ± 5 (6)[c]

[a] Final concentration in the assay of 20 μM except for captopril (10 nM). [b] Second contraction response to angiotensin I expressed as a percentage of the first contraction in the same arterial segment. Data are expressed as means ± SEM from *n* arterial segments. [c] Significantly different from the control ($P < 0.01$). [d] Significantly different from the control ($P < 0.05$).

Bradykinin is another naturally occurring ACE substrate with little structural resemblance to angiotensin I. In this study, peptides PACEI32 and PACEI34 (D- and L-stereoisomers) and $LfcinB_{17-31}$ were tested for their capacity to inhibit bradykinin degradation by ACE. None of them exhibited this capacity, confirming different mechanisms of ACE activity inhibition depending on the substrate and suggesting that the peptides interact with the angiotensin I site. In this regard, it has been reported that milk-derived inhibitor IC_{50} values were significantly lower when an angiotensin I-like substrate instead of a bradykinin-like substrate was used, suggesting that those inhibitors preferentially interfere with angiotensin metabolism (*35*). Moreover, in vivo experiments in mice demonstrated that the selective inhibition of either the N- or C-domain of ACE prevents the conversion of angiotensin I to angiotensin II, while bradykinin protection requires the inhibition of the two ACE active sites (*36*). Also, some studies suggest the possibility that ACE inhibitors beyond blocking bradykinin hydrolysis act through receptors (*31*).

Our ex vivo experiments showed that angiotensin I induces ACE-dependent contraction in the rabbit isolated carotid artery, as supported by the inhibition of angiotensin I-induced but not angiotensin II-induced contractions, by the ACE inhibitor captopril. This confirms previous results obtained in the same (*37*) and other vascular tissues (*37*, *38*). Local formation of angiotensin II from angiotensin I by ACE present in the arterial

wall is necessary for induction of contraction by angiotensin I, which lacks vasoactive effects by itself (*39*, *40*). Three peptides with in vitro inhibitory effects on ACE activity (PACEI32D, PACEI32L, and $LfcB_{17-31}$) showed ex vivo inhibitory effects on angiotensin I-induced contractions in the carotid artery, which adds functional evidence for the ACE inhibitory effects of these peptides in vascular tissue. No significant differences were found between PACEI32D and PACEI32L (**Table 5**). Quite interestingly, other peptides with in vitro ACE inhibitory effects (e.g., PACEI34L and PACEI34D) did not exhibit them ex vivo. This points to the importance of extending in vitro studies to functional studies.

All peptides used in this study have both termini protected which could be of interest in retaining its effects in vivo. In fact, peptides modified into cyclic form by a disulfide bond linking N- and C-terminal amino acids or modified with D-phenylglycine at the N-terminus retained the antihypertensive effect in the gastrointestinal tract and reduced the blood pressure in spontaneously hypertensive rats (*41*). We have shown that PACEIs synthesized with D-amino acids share ACE inhibitory properties both in vitro and ex vivo, which might be relevant for specific uses, as D-amino acid peptides are more stable and less susceptible to proteolytic degradation. In addition, the similar properties of sterically different peptides also indicate that the ACE inhibitory activity is more related to the identity of the side chains than to an overall structural conformation, as expected for these short peptides.

The data reported here from in vitro and ex vivo experiments demonstrate the ACE inhibiting effects of $LfcinB_{17-31}$ and PACEI32 in either configuration. Our work confirms and extends previously reported data on how minor amino acid changes affect biological properties (*19*). The stability of the three selected peptides against peptidases and their in vivo potential to modulate hypertension require further studies.

ABBREVIATIONS USED

ACE, angiotensin I-converting enzyme; LF, lactoferrin; LfcinB, lactoferricin B; HHL, hippuryl-L-histidyl-L-leucine; RP-HPLC, reversed-phase high-performance liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SEM, standard error of the mean; HSD, honestly difference procedure; SD, standard deviation.

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