

**INSTITUTO DE AGROQUÍMICA Y  
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**HOSPITAL UNIVERSITARIO  
Y  
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**UNIVERSIDAD POLITÉCNICA DE  
VALENCIA**  
Departamento de Tecnología de Alimentos



**EFFECTO ANTIHIPERTENSIVO,  
MEDIANTE INHIBICIÓN DE LA ENZIMA  
CONVERSORA DE ANGIOTENSINA I,  
DE PÉPTIDOS DERIVADOS DE LACTOFERRINA  
BOVINA Y PÉPTIDOS DISEÑADOS RACIONALMENTE**

**Memoria dirigida por:**

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**Presentada por:**

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**PARA OPTAR AL GRADO DE DOCTOR POR LA UNIVERSIDAD  
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**CERTIFICAN:** Que D. Pedro Ruiz Giménez, Licenciado en Ciencias Biológicas por la Universidad de Valencia, ha realizado bajo su codirección el trabajo titulado: “Efecto antihipertensivo, mediante inhibición de la enzima convertora de angiotensina I, de péptidos derivados de lactoferrina bovina y péptidos diseñados racionalmente”, que presenta para optar al grado de Doctor.

Y para que así conste a los efectos oportunos, firman el presente certificado en Valencia, 22 de Abril de 2013.

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## RESUMEN

En esta Tesis Doctoral se ha estudiado en modelos experimentales el potencial antihipertensivo de dos tipos de péptidos bioactivos: por un lado, péptidos derivados de distintas zonas de la secuencia de la lactoferrina bovina (LF), incluido su dominio antimicrobiano lactoferricina (LfcinB), y por otro lado heptapéptidos obtenidos mediante diseño racional a partir de hexapéptidos parentales. Se han realizado tres tipos de ensayos: ensayos *in vitro* para determinar los efectos inhibitorios de los péptidos sobre la actividad de la enzima convertora de la angiotensina I (ECA), utilizando diferentes sustratos artificiales y naturales; ensayos funcionales *ex vivo*, usando segmentos arteriales aislados de conejo, para analizar los efectos inhibitorios de los péptidos sobre la vasoconstricción ECA-dependiente producida por el sustrato natural angiotensina I (Ang I); y ensayos *in vivo* mediante la administración de los péptidos a ratas espontáneamente hipertensas (SHR) para estudiar los efectos antihipertensivos. En algunos casos, también se han realizado ensayos *in vitro* para determinar el potencial efecto tóxico de los péptidos no naturales en cultivos celulares y de digestión gastrointestinal simulada para analizar la biodisponibilidad de los péptidos.

Se obtuvieron péptidos derivados de LfcinB mediante diferentes elongaciones tanto del extremo C-terminal como del N-terminal del péptido representativo LfcinB<sub>20-25</sub> (RRWQWR). Estos péptidos mostraron diferentes potencias de inhibición de la actividad ACE *in vitro* y, excepto uno, inhibieron la vasoconstricción ECA-dependiente *ex vivo*. No se encontró una clara correlación entre los resultados *in vitro* y *ex vivo*. Solamente LfcinB<sub>20-25</sub> y un fragmento derivado (WQ) producido mediante digestión gastrointestinal simulada mostraron efectos antihipertensivos *in vivo*. Sin embargo, el fragmento no mostró efecto inhibitorio de la vasoconstricción ECA-dependiente en contraste con LfcinB<sub>20-25</sub>. Por otro lado, se preparó un hidrolizado de lactoferrina bovina con pepsina y se ultrafiltró para enriquecerlo en péptidos con peso molecular por debajo de 3 KDa

(LFH<3KDa). Este hidrolizado mostró efectos antihipertensivos mantenidos hasta 24 h tras la administración oral. LFH<3KDa se fraccionó mediante HPLC semi-preparativa, se identificaron 38 péptidos mediante espectrometría de masas en las fracciones inhibitoras de ECA y se sintetizaron los 11 péptidos más abundantes. Tres de estos péptidos (LIWKL, RPYL y LNNSRAP) mostraron diferentes grados de inhibición de la actividad ECA *in vitro* y efectos antihipertensivos *in vivo*, aunque solamente dos de ellos, LIWKL y RPYL, mostraron efectos inhibidores de la vasoconstricción ECA-dependiente *ex vivo*. Por último, seis heptapéptidos obtenidos mediante diseño racional mostraron diferentes grados de inhibición de la actividad ECA *in vitro* y de la vasoconstricción ECA-dependiente *ex vivo*, pero no de la vasoconstricción ECA-independiente producida por angiotensina II. Los heptapéptidos PACEI50L (RKWHFLW) y PACEI52L (RKWLFHW), así como el hexapéptido parental PACEI32L (RKWHFW), mostraron efectos antihipertensivos *in vivo*, sin afectar a la presión arterial en ratas normotensas. Los péptidos sintetizados con D-aminoácidos mostraron mucho menos efecto inhibidor de la actividad ECA *in vitro*, no tuvieron efecto *ex vivo*, y mostraron efectos antihipertensivos *in vivo* tras administración intravenosa pero no oral. La toxicidad de estos péptidos no naturales para reducir la viabilidad celular *in vitro* se mostró a concentraciones milimolares, mucho más altas que las concentraciones micromolares con efecto inhibidor de la actividad ECA.

En conclusión, se ha demostrado el potencial antihipertensivo de un péptido derivado del dominio antimicrobiano lactoferrina (LfcinB<sub>20-25</sub>), de un hidrolizado con pepsina de la lactoferrina enriquecido en péptidos de bajo peso molecular (LFH<3KDa), de péptidos contenidos en dicho hidrolizado procedentes de otras zonas de la lactoferrina diferentes de LfcinB (LIWKL, RPYL y LNNSRAP), y de hexa- y heptapéptidos obtenidos mediante diseño racional (PACEI32L, PACEI50L y PACEI52L). En la mayor parte de ellos, el efecto antihipertensivo se asocia a su efecto vasoactivo por su capacidad para inhibir la actividad ECA.



## ABSTRACT

In this Thesis we have studied the antihypertensive potential of two types of bioactive peptides in experimental models: peptides derived from different regions of the bovine lactoferrin (LF) sequence, including its antimicrobial domain lactoferricin B (LfcinB), and heptapeptides obtained by rational design based on two hexapeptides leads. Three types of assays have been carried out: peptides were assayed *in vitro* to determine their capacity to inhibit the angiotensin converting enzyme (ACE) activity using both synthetic and natural substrates; *ex vivo* functional assays in isolated rabbit arteries, to analyze inhibitory effects of peptides on ACE-dependent angiotensin I-induced vasoconstriction; and *in vivo* assays to study the antihypertensive effects of peptides administered to spontaneously hypertensive rats (SHR). In some cases, *in vitro* tests have been also carried out to discard cytotoxic effects of non natural peptides in cultured cells as well as simulated gastrointestinal digestion tests to analyse peptide bioavailability.

A set of LfcinB-derived peptides were obtained by different elongations both at the C-terminal and N-terminal ends of the representative peptide LfcinB<sub>20-25</sub> (RRWQWR). These peptides showed different *in vitro* inhibitory potencies on ACE activity and, except one of them, they showed *ex vivo* inhibitory effects on ACE-dependent vasoconstriction. No clear correlation between *in vitro* and *ex vivo* inhibitory effects was found. Only LfcinB<sub>20-25</sub> and one of its fragments (WQ), generated after a simulated gastrointestinal digestion, showed significant *in vivo* antihypertensive effects. However, the dipeptide WQ did not show any effect on ACE-dependent vasoconstriction in contrast to the inhibitory effect showed by LfcinB<sub>20-25</sub>. In addition, a bovine lactoferrin pepsin hydrolysate with molecular mass lower than 3KDa (LFH<3KDa) was prepared. This hydrolysate showed antihypertensive effects which remained significant for up to 24 h after oral administration. LFH<3KDa was further fractionated by

semi-preparative high performance liquid chromatography (HPLC), 38 peptides contained in the ACE-inhibitory fractions were identified by using ion trap mass spectrometry and, based on the peptide abundance, a total of 11 peptides were chemically synthesized. Three of them (LIWKL, RPYL and LNNSRAP) exerted different *in vitro* inhibitory effects on ACE and showed *in vivo* antihypertensive effects, although only two of them, LIWKL and RPYL, showed *ex vivo* inhibitory effect on ACE-dependent vasoconstriction. Finally, six heptapeptides obtained by rational design showed different degrees of *in vitro* ACE-inhibitory effect and *ex vivo* inhibitory effect on ACE-dependent vasoconstriction, but not inhibitory effect on ACE-independent angiotensin II-induced vasoconstriction. The heptapeptides PACEI50L (RKWHFLW) and PACEI52L (RKWLFHW), as well as the parental hexapeptide PACEI32L (RKWHFW) showed *in vivo* antihypertensive effects in SHRs, but they did not modify blood pressure of normotensive rats. When the D-amino acid enantiomeric peptides were used, the potency against *in vitro* ACE activity was strongly reduced, the inhibitory effect on *ex vivo* ACE-dependent vasoconstriction was lost and antihypertensive effect after intravenous administration but not after oral administration was shown. Cytotoxic potencies of the representative hexa- and heptapeptides assayed were much lower than their ACE-inhibitory potencies.

In conclusion, we have shown the antihypertensive potential of a LfcinB-derived peptide representative of the antimicrobial motif of LF (LfcinB<sub>20-25</sub>), of a pepsin hydrolysate of bovine LF enriched in low molecular weight peptides (LFH<3KDa), of peptides contained in this hydrolysate derived from LF regions other than LfcinB (LIWKL, RPYL and LNNSRAP), and of hexa- and heptapeptides obtained by rational design (PACEI32L, PACEI50L and PACEI52L). In most of them, the antihypertensive effect is in accordance with the vasoactive effect due to the inhibitory effect on ACE activity.

## RESUM

En esta Tesi Doctoral s'ha estudiat en models experimentals el potencial antihipertensiu de dos tipus de pèptids bioactius: d'una banda, pèptids derivats de diferents zones de la seqüència de la lactoferrina bovina (LF), inclòs el seu domini antimicrobià lactoferricina (LfcinB), i d'altra banda heptapèptids obtinguts mitjançant disseny racional a partir d'hexapèptids parentals. S'han realitzat tres tipus d'assajos: assajos *in vitro* per determinar els efectes inhibitoris dels pèptids sobre l'activitat de l'enzim conversor de la angiotensina I (ECA), utilitzant diferents substrats artificials i naturals; assajos funcionals *ex vivo*, usant segments arterials aïllats de conill, per analitzar els efectes inhibitoris dels pèptids sobre la vasoconstricció ECA-depenent produïda pel substrat natural angiotensina I (Ang I); i assajos *in vivo* mitjançant l'administració dels pèptids a rates espontàniament hipertenses (SHR) per estudiar els efectes antihipertensius. En alguns casos, també s'han realitzat assajos *in vitro* per determinar el potencial efecte tòxic dels pèptids no naturals en cultius cel·lulars i de digestió gastrointestinal simulada per analitzar la biodisponibilitat dels pèptids.

Es van obtenir pèptids derivats de LfcinB mitjançant diferents elongacions tant de l'extrem C-terminal com del N-terminal del pèptid representatiu LfcinB<sub>20-25</sub> (RRWQWR). Estos pèptids van mostrar diferents potències d'inhibició de l'activitat ECA *in vitro* i, excepte un, van inhibir la vasoconstricció ECA-depenent *ex vivo*. No es va trobar una clara correlació entre els resultats *in vitro* i *ex vivo*. Solament LfcinB<sub>20-25</sub> i un fragment derivat (WQ) produït mitjançant digestió gastrointestinal simulada van mostrar efectes antihipertensius *in vivo*. No obstant això, el fragment no va mostrar efecte inhibitor de la vasoconstricció ECA-depenent en contrast amb LfcinB<sub>20-25</sub>. D'altra banda, es va preparar un hidrolitzat de lactoferrina bovina amb pepsina i es va ultrafiltrar per enriquir-ho en pèptids amb pes molecular per baix de 3 KDa (LFH<3 KDa). Este hidrolitzat va

mostrar efectes antihipertensius mantinguts fins 24 h després de l'administració oral. LFH<3KDa es va fraccionar mitjançant HPLC semi-preparativa, es van identificar 38 pèptids mitjançant espectrometria de masses en les fraccions inhibidores de la ECA i es van sintetitzar els 11 pèptids més abundants. Tres d'estos pèptids (LIWKL, RPYL i LNNSRAP) van mostrar diferents graus d'inhibició de l'activitat ECA *in vitro* i efectes antihipertensius *in vivo*, encara que solament dos d'ells, LIWKL i RPYL, van mostrar efectes inhibidors de la vasoconstricció ECA-depenent *ex vivo*. Finalment, sis heptapèptids obtinguts mitjançant disseny racional van mostrar diferents graus d'inhibició de l'activitat ECA *in vitro* i de la vasoconstricció ECA-depenent *ex vivo*, però no de la vasoconstricció ECA-independent produïda per angiotensina II. Els heptapèptids PACEI50L (RKWHFLW) i PACEI52L (RKWLFHW), així com l'hexapèptid parental PACEI32L (RKWHFW), van mostrar efectes antihipertensius *in vivo*, sense afectar a la pressió arterial en rates normotenses. Els pèptids sintetitzats amb D-aminoàcids van mostrar molt menys efecte inhibidor sobre l'activitat ECA *in vitro*, no van tenir efecte *ex vivo*, i van mostrar efectes antihipertensius *in vivo* després de l'administració intravenosa però no oral. La toxicitat d'estos pèptids no naturals per reduir la viabilitat cel.lular *in vitro* es va mostrar a concentracions milimolars, molt més altes que les concentracions micromolars amb efecte inhibidor de l'activitat ECA.

En conclusió, s'ha demostrat el potencial antihipertensiu d'un pèptid derivat del domini antimicrobià lactoferrina (LfcinB<sub>20-25</sub>), d'un hidrolitzat amb pepsina de la lactoferrina enriquit en pèptids de baix pes molecular (LFH<3KDa), de pèptids continguts en dit hidrolitzat procedents d'altres zones de la lactoferrina diferents de LfcinB (LIWKL, RPYL i LNNSRAP), i de hexa- i heptapèptids obtinguts mitjançant disseny racional (PACEI32L, PACEI50L i PACEI52L). En la major part d'ells, l'efecte antihipertensiu s'associa al seu efecte vasoactiu per la seua capacitat per inhibir l'activitat ECA.

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## ABREVIATURAS

<i>Abz-Gly-Phe(NO<sub>2</sub>)-Pro</i>	<i>o-aminobenzoilglicil-p-nitrofenilalanilprolina</i>
ADH	<i>hormona antidiurética</i>
Ang I	<i>angiotensina I</i>
Ang II	<i>angiotensina II</i>
BPP	<i>péptidos potenciadores de la bradiquinina</i>
CO	<i>monóxido de carbono</i>
C-terminal	<i>carboxilo terminal</i>
ECA	<i>enzima convertora de la angiotensina I</i>
HHL	<i>hipuril-L-histidil-L-leucina</i>
IC <sub>50</sub>	<i>concentración de péptido necesaria para inhibir la actividad enzimática en un 50%.</i>
KCl	<i>cloruro potásico</i>
KDa	<i>KiloDaltons</i>
LF	<i>lactoferrina</i>
LFb	<i>lactoferrina bovina</i>
LfcinB	<i>lactoferricina bovina</i>
LFH	<i>hidrolizado de lactoferrina bovina</i>
ml	<i>mililitros</i>
mmHg:	<i>milímetros de mercurio</i>
NO	<i>óxido nítrico</i>

## ABREVIATURAS

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N-terminal	<i>amino terminal</i>
PACEIs	<i>péptidos inhibidores de la enzima convertora de angiotensina I</i>
RAS	<i>sistema renina-angiotensina</i>
RAT <sub>1</sub>	<i>receptores de angiotensina tipo I</i>
SHR	<i>rata espontáneamente hipertensa</i>
WKY	<i>rata Wistar-Kyoto</i>

Los diferentes aminoácidos se nombraron con el nombre completo cuando aparecían de manera individual y con el código de una letra cuando se encontraban en el interior de una secuencia peptídica.



## **INTRODUCCIÓN**



Los alimentos funcionales, definidos como aquellos que, además de satisfacer las necesidades nutricionales básicas, proporcionan beneficios para la salud o reducen el riesgo de sufrir enfermedades, están irrumpiendo con fuerza en los mercados internacionales dado el interés de los consumidores por la relación entre la alimentación y la salud. Sin embargo, las propiedades beneficiosas asociadas a este tipo de alimentos deben tener un sólido fundamento científico que además de proteger y dar confianza a los consumidores, ayude a la industria en el desarrollo, comercialización y promoción de estos productos.

El empleo de los péptidos bioactivos como ingredientes de los alimentos funcionales ha atraído la atención de los investigadores debido fundamentalmente a sus numerosas propiedades biológicas. Desde su descubrimiento a finales de la década de los 70, se han descrito péptidos derivados de proteínas alimentarias con diferentes actividades biológicas: opiácea, inmunomoduladora, antitrombótica, antimicrobiana, quelante de minerales y antihipertensiva, entre otras. Estos péptidos, inactivos en el interior de la secuencia de la proteína precursora, pueden liberarse por hidrólisis enzimática *in vitro* (durante el procesado de los alimentos) o *in vivo* (durante la digestión gastrointestinal).

De entre todas las posibles aplicaciones de los péptidos bioactivos, destaca la enfocada a la prevención y/o tratamiento de la hipertensión, dada su relación con las enfermedades cardiovasculares y la elevada incidencia de éstas en la población de los países desarrollados. En este contexto, se enmarcan los objetivos de la presente tesis, donde se evalúa la capacidad antihipertensiva de una serie de péptidos, tanto de origen alimentario como de nuevo diseño, mediante estudios *in vitro* de inhibición enzimática, *ex vivo* de vasoactividad en arterias aisladas e *in vivo* con un modelo animal de hipertensión.

## **1. Hipertensión arterial**

### **1.1. La hipertensión arterial como problema sanitario**

La hipertensión arterial se describe como una elevación crónica de la presión arterial. Se considera hipertensión valores de presión arterial media superiores a 110 mmHg en condiciones de reposo. Estos niveles ocurren cuando la presión arterial diastólica es superior a 90 mmHg y la sistólica mayor de 140 mmHg.

El incremento del riesgo cardiovascular inherente a la hipertensión provoca una prematura morbilidad y mortalidad (Giles y cols., 2009; Ricci y cols., 2010). Por ello la hipertensión arterial supone un serio problema sanitario en los países desarrollados tanto desde el punto de vista económico, generando un elevado coste para la sociedad, como médico, ya que si no es tratada el riesgo de producirse complicaciones cardio y cerebrovasculares como son el infarto de miocardio, ictus y también lesiones renales es de 2 a 3 veces mayor que en individuos normotensos (Kannel, 1996). Diversos estudios clínicos (Collins y cols., 1990; MacMahon y cols., 1990) han demostrado que reducciones de la presión arterial prolongadas pero relativamente pequeñas de sólo 6 mmHg en la presión sistólica y entre 5 y 10 mmHg en la presión diastólica reducen el riesgo de infarto (30-40%) y de enfermedad coronaria (20%). Es por esto que tratamientos farmacológicos o cambios en el estilo de vida (reducción del sobrepeso, evitar hábitos nocivos como el tabaco, evitar el estrés y adoptar hábitos alimentarios saludables) que provoquen una bajada de valores de presión arterial tienen un considerable impacto sobre la hipertensión y el consiguiente riesgo de enfermedades cardiovasculares (Rashid y cols., 2003; Boelsma y Kloek, 2009; Williams, 2009).

## **1.2. Mecanismos de control de la presión arterial: sistema renina-angiotensina (RAS)**

Los mecanismos de control de la presión arterial son diversos y están interrelacionados entre sí. Por una parte está el control a corto plazo de la presión arterial, muy rápido y llevado a cabo por el sistema nervioso, que consiste en el control del gasto cardíaco y la resistencia periférica. Por otro lado, para la regulación prolongada de la presión arterial se requiere de otros sistemas de control, principalmente el sistema de control renal y de los líquidos corporales así como de los mecanismos hormonales de los que forman parte el sistema renina-angiotensina (RAS), el sistema quinina-calitreína, el sistema de la endopeptidasa neutra y el sistema de la enzima convertora de la endotelina (Guyton, 1996; Fitzgerald y cols., 2004).

El funcionamiento del RAS, que se esquematiza en la Figura 1, es el siguiente: cuando la presión arterial disminuye, se libera al torrente sanguíneo la renina, una proteasa sintetizada principalmente en el riñón. En los vasos sanguíneos, la renina actúa sobre el angiotensinógeno, péptido de 14 aminoácidos sintetizado en el hígado, liberando un decapeptido, la angiotensina I (Ang I) de secuencia DNVYIHPFHL. A continuación, se escinde el dipéptido carboxilo terminal (C-terminal) de la Ang I por la acción de la enzima convertora de la angiotensina I (ECA) formándose el octapéptido denominado angiotensina II (Ang II), DNVYIHPF, que permanece en la sangre hasta ser inactivada por un conjunto de enzimas denominadas angiotensinasas.

## Introducción

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Durante su permanencia en la sangre, la Ang II provoca una serie de efectos que elevan la presión arterial:

a) La Ang II se une a los receptores de angiotensina tipo I ( $RAT_1$ ) situados en el músculo liso, produciéndose una vasoconstricción de las arteriolas, aumentando la resistencia periférica total y la presión arterial. Este efecto se produce durante los segundos posteriores a su síntesis.

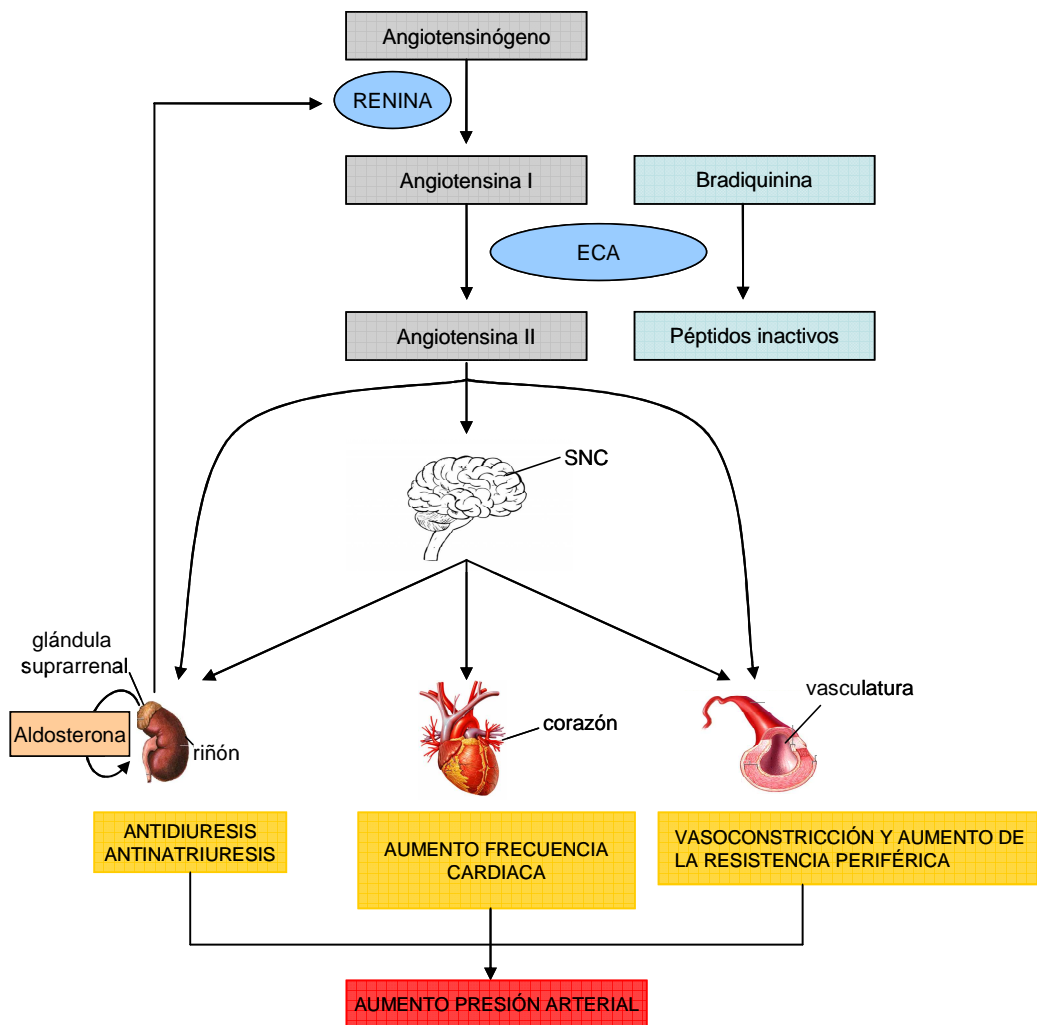
b) La unión de la Ang II a los receptores situados en la corteza suprarrenal estimula la liberación de aldosterona. La aldosterona provoca un aumento de la reabsorción de sal y agua por parte de los túbulos renales. Esta disminución de la excreción de sal y agua aumenta el volumen de líquido extracelular, provocando el aumento de la presión arterial a lo largo de un período cuya duración puede oscilar entre horas y días.

c) La Ang II actúa también a nivel del sistema nervioso central. Desencadena el mecanismo de la sed en el hipotálamo a través de la estimulación de la secreción de la hormona antidiurética (ADH), estimulándose a su vez el "apetito" por la sal. La mayor ingesta de agua y sal aumentan el volumen extracelular restableciéndose los valores de presión arterial.

d) Por último, la Ang II actúa sobre el sistema nervioso autónomo, de manera que la interacción Ang II-receptor en las neuronas noradrenérgicas aumenta la frecuencia cardíaca y la resistencia periférica provocando un aumento de la presión arterial.

Además de la formación de la Ang II, la ECA posee una segunda función importante para la regulación de la presión arterial como es la hidrólisis de la bradiquinina, vasodilatador que forma parte del sistema quinina-caliceína. Por lo tanto, a los efectos del aumento de la presión arterial contribuyen la generación de un vasopresor como la Ang II y la hidrólisis de un vasodilatador como la bradiquinina.

Por todo ello, el RAS, aunque no es el único mecanismo humoral implicado en el control de la presión arterial, está considerado como uno de sus principales reguladores (FitzGerald y cols., 2004), por lo que gran parte de los estudios dirigidos al tratamiento de la hipertensión se centran en este sistema, y más concretamente en uno de sus componentes principales, la enzima convertidora de la angiotensina I (ECA).



**Figura 1.** Esquema del funcionamiento del RAS en la regulación de la presión arterial

### **1.2.1. Enzima Conversora de la Angiotensina I (ECA)**

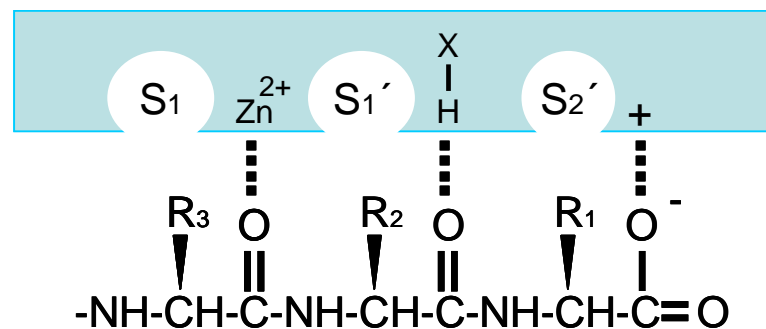
La ECA también conocida como peptidil-dipeptidasa A (EC 3.4.15.1) es uno de los principales componentes del RAS. La conservación estructural y funcional que presenta el gen que la codifica indica su importancia dentro de los mecanismos de regulación de la presión arterial (Acharya y cols., 2003). La ECA forma parte de la familia M2 de las metalopeptidasas de zinc, con 2 dominios (dominio carboxilo terminal y dominio amino terminal) cada uno con un centro catalítico y con similares aunque distintas especificidades de sustrato.

La ECA se purificó parcialmente por primera vez a partir de plasma de caballo y los estudios llevados a cabo sugerían que contenía un ión metálico fuertemente unido, ya que su actividad era inhibida por agentes quelantes de metales (Skeggs y cols., 1956). Estudios posteriores mostraron que la ECA es un glicoproteína formada por una sola cadena polipeptídica con un peso molecular entre 130 y 150 KDa (Das y Soffer, 1975; Soffer, 1976; Lanzillo y Fanburg, 1976).

Durante la década de los 70 se estableció que la ECA es una dipeptidil carboxipeptidasa que cataliza la hidrólisis de dipéptidos del extremo C-terminal de una amplia variedad de oligopéptidos, entre los que se incluyen la Ang I y la bradiquinina. Fue también durante esta década cuando los estudios de especificidad de sustrato utilizando tripéptidos permitieron establecer que un aminoácido aromático como la fenilalanina en la antepenúltima posición de un sustrato peptídico contribuye de manera fundamental a la afinidad por la enzima. Por el contrario, péptidos como la Ang II con un residuo de prolina en la penúltima posición, aquellos con un residuo dicarboxílico en posición terminal como el glutámico o aquellos sin un grupo C-terminal ionizable no son hidrolizados por la ECA. Estos requerimientos estructurales eran bastante similares a los observados para la carboxipeptidasa A de páncreas bovino, lo que sugería que la ECA era una exopeptidasa con un mecanismo hidrolítico similar al de las carboxipeptidasas (Cushman y cols., 1981).



Estos primeros estudios de especificidad de sustrato, así como trabajos posteriores, indican que los sustratos peptídicos se unen al sitio activo de la enzima predominantemente a través del tripéptido C-terminal. La región del sitio activo a la cual se une el tripéptido C-terminal se designa como sitio de unión obligatorio, tal y como se describe en la Figura 2. En él destacan los subsitios  $S_1$ ,  $S_1'$  y  $S_2'$  donde específicamente interaccionan las cadenas laterales de los aminoácidos. El subsitio  $S_1$  es un bolsillo hidrofóbico con una buena afinidad por las cadenas aromáticas como las de la fenilalanina, el subsitio  $S_1'$  no interacciona de manera efectiva con la prolina, mientras que el subsitio  $S_2'$  no acepta las cadenas ácidas de los aminoácidos dicarboxílicos. Estos estudios también establecieron que en el sitio activo de la enzima existe un grupo cargado positivamente que forma un enlace iónico con el grupo C-terminal del sustrato cargado negativamente lo que provoca el alineamiento correcto del tripéptido C-terminal del sustrato con los subsitios de la ECA. Así mismo, se estableció que el ión  $Zn^{2+}$  de la ECA se localizaba entre los subsitios  $S_1$  y  $S_1'$  y que participaba en la hidrólisis del enlace peptídico entre los residuos antepenúltimo y penúltimo del sustrato, liberándose el dipéptido C-terminal (Cushman y cols., 1981; Ondetti y Cushman, 1982).



**Figura 2.** Modelo de interacción del centro activo de la ECA con los sustratos o inhibidores competitivos

### 1.2.2. Inhibidores de la ECA

Los primeros péptidos inhibidores de la ECA se aislaron a partir del veneno de la serpiente *Bothrops jararaca* (Ondetti y cols., 1971; Cheung y Cushman, 1973). Estos péptidos, que poseían desde cinco hasta trece residuos aminoacídicos, habían sido previamente descritos por Ferreira y colaboradores (1970) como péptidos potenciadores de la bradiquinina (BPP) (Tabla 1), ya que inhibían la degradación de la misma en plasma (Ferreira, 1966). De ellos, los dos péptidos más potentes *in vitro* resultaron ser el pentapéptido de secuencia EKWAP, y el nonapéptido EWPRPQIPP. Estudios *in vivo* demostraron que el pentapéptido era rápidamente metabolizado por peptidasas mientras que el nonapéptido, mucho más estable, mostró efecto antihipertensivo en animales modelo, aunque sólo tras administración parenteral.

Los estudios de estructura-actividad llevados a cabo con estos dos péptidos inhibidores y diversos análogos, demostraron que compiten con los sustratos de la enzima por unirse a los mismos sitios de unión ( $S_1$ ,  $S_1'$  y  $S_2'$ ). Además, sólo los tripéptidos del extremo C-terminal del pentapéptido y el nonapéptido mantenían la capacidad de inhibición de la ECA (Cushman y cols., 1973; Cheung y Cushman, 1973). La sustitución de ciertos residuos de aminoácidos en el tripéptido C-terminal, demostró, que al igual que en el caso de los sustratos, un aminoácido aromático en la antepenúltima posición favorece la unión a la enzima, mientras que una prolina en penúltima posición así como un aminoácido dicarboxílico en posición terminal inhibían la unión. En concreto, las secuencias WAP y FAP fueron seleccionadas como las óptimas para unirse a los sitios de unión de la ECA.

Aunque el nonapéptido aislado a partir de veneno de serpiente presentaba efecto antihipertensivo por vía parenteral en animales modelo, perdía su efectividad al ser administrado por vía oral. Por ello, se desarrollaron nuevos inhibidores no peptídicos análogos a los péptidos naturales, diseñados para interactuar con el centro activo de la enzima con

la máxima eficacia y ser efectivos por vía oral. Entre ellos se encuentran los mercaptoalcanoil aminoácidos, destacando entre ellos el captopril (D-3-mercapto-2-metilpropanoil-L-prolina). Este inhibidor es un análogo de la secuencia Alanina-Prolina con un grupo sulfidrilo adicional. La unión al centro activo de la ECA es similar al descrito para los sustratos y péptidos inhibidores naturales, con una interacción adicional del grupo sulfidrilo con el ión  $Zn^{2+}$  del centro catalítico de la enzima lo que incrementa su potencia inhibidora (Ondetti y Cushman, 1982). El captopril, un potente agente terapéutico en el tratamiento de la hipertensión, fue el primer inhibidor de la ECA de uso clínico. Al captopril le han seguido otros inhibidores, un total de 17 hasta el año 2002, que han sido aprobados para su uso clínico, y que son variaciones del captopril diseñadas para mejorar la interacción con el  $Zn^{2+}$  y el subsitio  $S_2'$  (Acharya y cols., 2003).

**Tabla 1.** Primeros péptidos inhibidores de la ECA aislados a partir del veneno de la serpiente *Bothrops jararaca* [Adaptada de Cushman y cols., 1981]

Péptido <sup>1</sup>	Secuencia
BPP <sub>5a</sub>	EKWAP
BPP <sub>9a</sub>	EWPRPQIPP
BPP <sub>10a</sub>	ESWPGPNIPP
BPP <sub>10b</sub>	ENWPRPQIPP
BPP <sub>10c</sub>	ENWPHPQIPP
BPP <sub>11d</sub>	EWPRPTPQIPP
BPP <sub>13a</sub>	EGGWPRPGPEIPP

<sup>1</sup> La nomenclatura de los péptidos con las siglas **BPP** se corresponden con la abreviatura en inglés de **B**radykinin **P**otentiating **P**eptide.

## **2. Péptidos bioactivos con capacidad antihipertensiva**

A pesar del abanico de fármacos antihipertensivos disponible, y de la elevada eficacia que poseen la mayor parte de ellos, existe el problema de los efectos secundarios que pueden producir, algunos de ellos importantes, como cuadros de hipotensión y de tos, pérdidas del sentido del gusto, fatiga, erupciones cutáneas e incluso malformaciones congénitas (Atkinson y Robertson, 1979).

Esta problemática ha hecho que se sigan buscando compuestos que sean a la vez específicos contra la hipertensión y que reduzcan los efectos secundarios. En la actualidad, la búsqueda de péptidos bioactivos derivados de proteínas alimentarias que puedan presentar actividad antihipertensiva ha despertado gran interés, ya que podrían constituir una alternativa más natural para la prevención y/o tratamiento de la hipertensión.

Los péptidos bioactivos son generalmente de pequeño tamaño, entre 2 y 15 residuos de aminoácidos, y forman parte de la secuencia de la proteína alimentaria de manera que éstas, además de su valor nutricional y de su valor funcional afectando a las propiedades físico-químicas y organolépticas de los alimentos, poseen un papel fisiológico como fuente de péptidos bioactivos.

Se han descrito numerosos péptidos con actividad inhibidora de la ECA obtenidos de proteínas alimentarias tanto de origen animal como vegetal (Pihlanto y Korhonen, 2003; Murray y FitGerald, 2007; Li y cols., 2004; Hong y cols., 2008; De Leo y cols., 2009). Entre los de origen vegetal destacan los péptidos obtenidos a partir de hidrolizados de soja (Wang y González de Mejia, 2005; Koderá y cols., 2006) y de sake (Saito y cols., 1994). También se han identificado péptidos con actividad inhibidora de la ECA en hidrolizados de músculo de diversos animales como el bonito, salmón, sardina, atún, gallina y cerdo, entre otros (Vercruyssen y cols., 2005), a partir de hidrolizados de proteínas del huevo como la ovoalbúmina

y la ovotransferrina (Miguel y cols., 2006; Miguel y cols., 2007a), y a partir de hidrolizados de proteínas lácteas (Kohmura y cols., 1989; Ricci y cols., 2010). En la actualidad, la fuente principal de péptidos antihipertensivos son las proteínas lácteas, destacando los péptidos derivados de caseína (Silva y cols., 2005; Miguel y cols., 2009).

### **2.1. Péptidos antihipertensivos derivados de proteínas lácteas**

Durante la última década, se han publicado diversos artículos de revisión sobre las propiedades biológicas de péptidos derivados de proteínas lácteas (Korhonen y cols., 2006), entre las que destaca la capacidad antihipertensiva (Fitzgerald y cols., 2004; López-Fandiño y cols., 2006; Martínez-Maqueda y cols., 2012).

La estrategia más común para caracterizar los péptidos antihipertensivos incluye ensayos de inhibición de la ECA *in vitro* y experimentos *in vivo* con animales. Para los primeros, se han desarrollado métodos analíticos rápidos, sensibles y fiables, basados generalmente en ensayos espectrofotométricos y cromatográficos (Cushman y Cheung, 1971; Vermeirssen y cols., 2002; Wu y cols., 2002; Li y cols., 2005; Sentandreu y Toldrá, 2006), donde se determina la actividad ECA sobre un sustrato apropiado en presencia y ausencia del potencial péptido inhibidor. La potencia inhibitoria se expresa como valor de  $IC_{50}$ , que se define como la concentración de péptido necesaria para inhibir la actividad enzimática en un 50%. Para los experimentos *in vivo* se utilizan ratas espontáneamente hipertensas (SHR) que constituyen un modelo animal científicamente aceptado para estudiar la hipertensión (Fitzgerald y cols., 2004). Este tipo de estudios se aplica para evaluar los efectos de los potenciales péptidos antihipertensivos tanto en ensayos de administración aguda como crónica. Además, estos estudios suelen incluir la evaluación del efecto de los péptidos sobre la presión arterial de ratas normotensas Wistar-Kyoto (WKY).

## Introducción

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Los péptidos antihipertensivos derivados de proteínas lácteas son producidos principalmente por hidrólisis enzimática o mediante fermentación con bacterias lácticas. Su evaluación *in vitro* e *in vivo* ha puesto de manifiesto la falta de correlación entre los resultados obtenidos con ambos tipos de ensayos. De hecho, a pesar del ingente número de péptidos derivados de proteínas lácteas con actividad inhibidora *in vitro* (ver revisión Ricci y cols., 2010), sólo una pequeña proporción de ellos presenta efecto antihipertensivo *in vivo*. En la Tabla 2 se recogen los péptidos con efecto antihipertensivo derivados de caseínas y proteínas del suero obtenidas bien mediante hidrólisis enzimática o fermentación.

En los siguientes epígrafes se resumen los péptidos identificados a partir de hidrolizados enzimáticos de proteínas lácteas o de productos lácteos fermentados, con especial incidencia en aquellos que han sido evaluados en ensayos clínicos.

**Tabla 2.** Péptidos con efecto antihipertensivo derivados de caseínas y proteínas del suero obtenidos mediante hidrólisis enzimática o fermentación [adaptada de Hernández-Ledesma y cols., 2011]

Fragmento	Secuencia	IC <sub>50</sub> <sup>a</sup>	PAS <sup>b</sup>	Origen	Referencia
α <sub>s1</sub> -CN <sup>c</sup> f(1-9)	RPKHPIKHQ	13.4	-9.3	Queso Gouda	Saito y cols., 2000
α <sub>s1</sub> -CN f(102-109)	KKYNVPQL	77.1	-11.5	Queso Manchego	Gómez-Ruiz y cols., 2002
α <sub>s1</sub> -CN f(146-147)	YP	720.0	-32.1	Fermentación con <i>L.helveticus</i> CPN4	Yamamoto y cols., 1999
β-CN f(58-68)	LVYFPFGPIPN	27.9	-28.0	Kefir de leche de cabra	Miguel y cols., 2010
β-CN f(58-76)	LVYFPFGPIPNSLPQNIPP	5.2	-15.0	Fermentación con <i>E.faecalis</i>	Quirós y cols., 2007
β-CN f(59-68)	VYFPFGPIPN	14.8	-7.0	Queso Gouda	Saito y cols., 2000
β-CN f(60-68)	YFPFGPIPN	14.8	-7.0	Queso Gouda	Saito y cols., 2000
β-CN f(74-76)	IPP	5.0	-28.3	Fermentación con <i>L.helveticus</i> y <i>S.cerevisiae</i>	Nakamura y cols., 1995a y b
β-CN f(84-86)	VPP	9.0	-32.1	Fermentación con <i>L.helveticus</i> y <i>S.cerevisiae</i>	Nakamura y cols., 1995a y b
β-CN f(133-138)	LHLPLP	5.5	-21.9	Fermentación con <i>E.faecalis</i>	Quirós y cols., 2007
β-CN f(197-206)	VLGPVRGPPF	137.0	-16.2	Fermentación con <i>E.faecalis</i>	Quirós y cols., 2007
β-CN f(201-209)	VRGPFPIIV	599.0	-16.1	Fermentación con <i>E.faecalis</i>	Quirós y cols., 2007
α <sub>s1</sub> -CN f(23-34)	FFVAPFPGVFGK	77.0	-34.0	Hidrólisis con tripsina	Karaki y cols., 1990
α <sub>s1</sub> -CN f(90-94)	RYLGY	0.7	-25.0	Hidrólisis con pepsina	Contreras y cols., 2009
α <sub>s1</sub> -CN f(143-149)	AYFYPEL	6.6	~ -21	Hidrólisis con pepsina	Contreras y cols., 2009
α <sub>s2</sub> -CN f(189-95)	YQKFPQY	20.1	~ -15	Hidrólisis con pepsina	Contreras y cols., 2009
α <sub>s2</sub> -CN f(203-208)	PYVRYL	1.9	-23.4	Hidrólisis con pepsina	Recio y cols., 2005
β-CN f(59-61)	VYP	288.0	-21.0	Hidrólisis con proteinasa k	Abubakar y cols., 1998

(continúa en página siguiente)

**Tabla 2** (continuación)

Fragmento	Secuencia	IC <sub>50</sub> <sup>a</sup>	PAS <sup>b</sup>	Origen	Referencia
β-CN f(59-64)	VYFPFG	221.0	-22.0	Hidrólisis con proteinasa k	Abubakar y cols., 1998
β-CN f(169-174)	KVLPVP	5.0	-32.2	Hidrólisis con proteinasa de <i>L.helveticus</i> CP790	Maeno y cols., 1996
β-CN f(169-175)	KVLPVPQ	1000.0	-31.5	Hidrólisis con proteinasa de <i>L.helveticus</i> CP790	Maeno y cols., 1996
κ-CN f(22-24)	IAK	15.7	-20.7	Hidrólisis con enzimas digestivos	Miguel y cols., 2010
κ-CN f(61-66)	YAKPVA	14.3	-23.1	Hidrólisis con enzimas digestivos	Miguel y cols., 2010
κ-CN f(76-86)	WQVLPNAVPAK	10.1	-18.4	Hidrólisis con enzimas digestivos	Miguel y cols., 2010
κ-CN f(98-105)	HPHPHLSF	28.9	-15.7	Hidrólisis con enzimas digestivos	Miguel y cols., 2010
κ-CN f(106-112)	MAIPPKK	4785.0	~ -28	Hidrólisis con tripsina	Miguel y cols., 2007b
α-La <sup>d</sup> f(50-53)	YGLF	733.0	-23.0	Hidrólisis con enzimas gástricos y pancreáticos	Nurminen y cols., 2000
β-Lg <sup>e</sup> f(58-61)	LQKW	34.7	-18.1	Hidrólisis con termolisina	Hernández-Ledesma y cols., 2007
β-Lg f(78-80)	IPA	141.0	-31.0	Hidrólisis con proteinasa k	Abubakar y cols., 1998
β-Lg f(103-105)	LLF	79.8	~ -20	Hidrólisis con termolisina	Hernández-Ledesma y cols., 2007
β-Lg f(142-145)	ALPM	928.0	-21.4	Producto comercial de proteínas del suero	Murakami y cols., 2004
BSA <sup>f</sup> f(221-222)	FP	315.0	-27.0	Hidrólisis con proteinasa k	Abubakar y cols., 1998

*L.helveticus*: *Lactobacillus helveticus*; *S. cerevisiae*: *Saccharomyces cerevisiae*; *E. faecalis*: *Enterococcus faecalis*

IC<sub>50</sub><sup>a</sup>: concentración (μM) necesaria para inhibir el 50% de la actividad de la ECA.

PAS<sup>b</sup>: disminución de la presión arterial sistólica (mm Hg) después de la administración oral a ratas espontáneamente hipertensas.

CN<sup>c</sup>: caseína.

La<sup>d</sup>: lactoalbúmina

Lg<sup>e</sup>: lactoglobulina

BSA<sup>f</sup>: seroalbúmina bovina



### **2.1.1. Hidrólisis enzimática**

La hidrólisis enzimática es la estrategia más común para la obtención de péptidos bioactivos a partir de proteínas alimentarias. Se lleva a cabo empleando enzimas implicadas en la digestión gastrointestinal como la pepsina del estómago y la tripsina pancreática, simulando las condiciones fisiológicas (pH y temperatura) de la digestión *in vivo*. También se han utilizado enzimas de origen vegetal como la bromelaína, y de origen microbiano como la proteínasa K y la termolisina (López-Fandiño y cols., 2006).

La hidrólisis enzimática permite la selección de la proteína a hidrolizar, de la enzima específica para llevar a cabo la hidrólisis, así como de las condiciones del proceso (pH, temperatura y tiempo), lo cual optimiza la obtención de péptidos bioactivos. Además, este proceso puede optimizarse aún más mediante técnicas de enriquecimiento de las fracciones y de aislamiento de los péptidos deseados dentro del hidrolizado total, para lo que se utilizan técnicas de precipitación, ultrafiltración y cromatografía. Las técnicas de precipitación se suelen utilizar con el objeto de eliminar proteínas, enzimas y otros componentes del hidrolizado inicial, aunque también como método de aislamiento de péptidos bioactivos siempre que éstos posean un grupo funcional particular que haga esta precipitación selectiva. Por ejemplo los péptidos fosforilados derivados de la caseína (Adamson y Reynolds, 1995). En el caso de que los péptidos buscados posean unos tamaños particulares, como es el caso de los péptidos inhibidores de la ECA, la mayoría de los cuales poseen tamaños menores a 3 KDa, se puede utilizar la ultrafiltración o la cromatografía de exclusión molecular (López-Fandiño y cols., 2006). Otra característica de los péptidos inhibidores de la ECA es su hidrofobicidad, por lo que otra técnica utilizada para su aislamiento es la cromatografía en fase inversa (Manso y López-Fandiño, 2003).

## Introducción

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Existen numerosos ejemplos de péptidos derivados de proteínas lácteas aislados a partir de hidrolizados enzimáticos con efecto antihipertensivo (ver Tabla 2). Por lo que se refiere a su efecto en humanos, destacan los estudios realizados con el producto denominado Dodecapeptide® (Soft Drink-Casein DP Peptio Drink, Japan), péptido aislado a partir de un hidrolizado enzimático de caseína. Este péptido mostró una reducción de la presión diastólica de 6 mmHg cuando se administró durante 4 semanas (Sugai, 1998) y una disminución de la presión sistólica de 9.2 mmHg en un estudio con administración aguda en el que se midió la presión arterial 6 horas después de la administración (Townsend y cols., 2004). Asimismo, Cadee y cols. (2007) obtuvieron disminuciones significativas tanto de la presión sistólica como diastólica administrando durante 4 semanas un hidrolizado de caseína que contenía este péptido.

También se han realizado estudios clínicos con hidrolizados completos de caseína, como el descrito por Mizuno y cols. (2004) que fue obtenido utilizando una preparación proteolítica de *Aspergillus oryzae*. Este hidrolizado, que mostró un efecto antihipertensivo en ratas SHR y que contiene los tripéptidos IPP y VPP, es comercializado por Calpis como AmealPeptide® y ha sido evaluado en estudios clínicos donde ha demostrado su eficacia (Mizuno y cols., 2005; Sano y cols., 2005). Otro producto que ha demostrado la eficacia en humanos es un hidrolizado procedente de proteínas del suero de leche, BioZate 1® (Davisco, USA) (Pins y Keenan, 2003). Recientemente, se ha demostrado la eficacia de un hidrolizado de caseína en pacientes hipertensos, cuya presión arterial descendió 12.5 mmHg tras un tratamiento de 6 semanas (Recio y cols., 2011). Este hidrolizado contiene dos fragmentos derivados de la  $\alpha_{S1}$ -caseína, previamente caracterizados como inhibidores de la ECA y con efecto antihipertensivo en ratas SHR (Contreras y cols., 2009).

### **2.1.2. Procesos fermentativos**

Durante la fermentación de productos lácteos, las bacterias ácido-lácticas hidrolizan las proteínas, principalmente la caseína, originando péptidos y aminoácidos que utilizarán como fuente de nitrógeno para su crecimiento (Juillard y cols., 1998). El sistema proteolítico de *Lactobacillus helveticus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactococcus lactis* ssp. *diacetylactis*, *L. lactis* ssp. *cremoris* y *Streptococcus salivarius* ssp. *thermophilus* ha demostrado su capacidad para liberar péptidos inhibidores de la ECA (Fitzgerald y Murray, 2006; Korhonen y Pihlanto, 2006; López-Fandiño y cols., 2006). Asimismo, otras especies bacterianas como *Enterococcus faecalis* son capaces de liberar péptidos a partir de la  $\beta$ -caseína, con capacidad inhibitoria de la ECA y efecto antihipertensivo en ratas SHR tras administración oral en ensayos agudos y crónicos (Miguel y cols., 2006; Quirós y cols., 2007). Además de en leches fermentadas, también se producen péptidos inhibidores de la ECA durante el proceso de maduración de los quesos (Saito y cols., 2000; Miguel y cols., 2010). En la Tabla 2 se muestran los péptidos producidos durante procesos de fermentación que han mostrado efecto antihipertensivo en ratas SHR.

Sin lugar a dudas, los lactotripéptidos VPP e IPP producidos en leches fermentadas con *L. helveticus* (Nakamura y cols., 1995a; Nakamura y cols., 1995b) y también identificados en diferentes variedades de quesos (Butikofer y cols., 2007; Butikofer y cols., 2008) son los péptidos antihipertensivos mejor caracterizados. Las leches fermentadas que los contienen, comercializadas en Japón (Calpis/AmealS®, Calpis Co. Ltd., Tokio, Japón) y Finlandia (Valio Evolus® Double Effect, Valio Ltd., Finlandia) han mostrado efectos beneficiosos sobre la presión arterial en varios modelos animales y en humanos.

## Introducción

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Los ensayos clínicos realizados con Calpis®, muestran que los valores de presión sistólica disminuyen una media de 14.1 mmHg después de la administración oral durante 8 semanas de 95 mL de la leche fermentada (Hata y cols., 1996) y de 5.2 mmHg cuando la administración se llevó a cabo durante 4 semanas (Mizushima y cols., 2004). En el caso de Evolus®, diferentes estudios han demostrado disminuciones de la presión sistólica en pacientes hipertensos como por ejemplo disminuciones de 14.9 mmHg tras una administración de 8 semanas (Seppo y cols., 2002) y de 6.7 mmHg cuando se administró durante 21 semanas (Seppo y cols., 2003). Además de las dos leches fermentadas ya citadas, se han abordado estudios clínicos con diferentes productos que contienen los tripéptidos (Itakura y cols., 2001; Kajimoto y cols., 2002; Jauhiainen y cols., 2005).

Los resultados de muchos de los ensayos clínicos citados se recogen en dos trabajos donde se realiza un meta-análisis de los mismos (Pripp, 2008; Xu y cols., 2008). El descrito por Xu y colaboradores (2008) incluye 12 ensayos clínicos con un total de 623 participantes y observa descensos significativos en la presión arterial sistólica y diastólica (4.8 mmHg y 2.2 mmHg, respectivamente). Resultados similares se obtuvieron en el meta-análisis realizado por Pripp (2008) con un total de 15 ensayos clínicos. Sin embargo, también hay estudios clínicos donde no se observa ningún efecto sobre la presión arterial tras la administración crónica de los tripéptidos (Hirota y cols., 2007; Engberink y cols., 2008; Van der Zander y cols., 2008; Usinger y cols., 2009; Van Mierlo y cols., 2009), poniendo de manifiesto la influencia de factores tales como la composición del producto administrado, la dosis y la metodología para la medida de la presión arterial en los resultados obtenidos (Boelsma y Kloek, 2009; Jakala y Vapaatalo, 2010).

A modo de resumen, se muestran en la Tabla 3 los ensayos clínicos realizados para estudiar los efectos antihipertensivos de la administración de leches fermentadas, hidrolizados proteicos o péptidos.

**Tabla 3.** Estudios clínicos sobre el efecto antihipertensivo de hidrolizados proteicos y leches fermentadas [adaptada de Hernández-Ledesma y cols., 2011]

Producto	Dosis	Duración del tratamiento (semanas)	Efecto en la PAS <sup>1</sup> (mm Hg)	Referencias
Dodecapeptide® <sup>2</sup>	200 mg de FFVAPFPEVFGK	dosis única	-9.2	Townsend y cols., 2004
Dodecapeptide®	239.4 mg FFVAPFPEVFGK	4	-10.7	Cadee y cols., 2007
AmealPeptide® <sup>3</sup>	1.8 mg IPP + VPP	6	-6.3	Mizuno y cols., 2005
AmealPeptide®	2.5 mg IPP + VPP	6	-6.7	Mizuno y cols., 2005
AmealPeptide®	3.6 mg IPP + VPP	6	-10.1	Mizuno y cols., 2005
AmealPeptide®	200 mL/día (1.6 mg IPP + 1.5 mg VPP)	12	-9.5	Sano y cols., 2005
BioZate 1® <sup>4</sup>	no determinada	6	-11.0	Pins y Keenan, 2003
Aislado de proteínas de suero hidrolizadas con tripsina y mezclas con cacao y aspartamo	20 g/día	6	-8.0	Pins y Keenan, 2006
Yogur conteniendo un hidrolizado de caseína por <i>Aspergillus oryzae</i>	200 g/día (10.2 mg de IPP+VPP)	8	N.S. <sup>5</sup>	Van der Zander y cols., 2008
Cápsulas conteniendo un hidrolizado de caseína por <i>Aspergillus oryzae</i>	4 cápsulas (3.9 mg IPP + 8.3 mg VPP)	1	N.S. <sup>5</sup>	Hirota y cols., 2007
Yogur conteniendo un hidrolizado de caseína con pepsina	150 mL (2.5-3.1 mg de hidrolizado)	6	-12.5	Recio y cols., 2011
Calpis/Ameal S® <sup>6</sup>	95 mL/día (1.1 mg IPP + 1.5 mg VPP)	8	-14.1	Hata y cols., 1996
Calpis/Ameal S®	160 g/día(1.2 mg IPP + 2.0 mg VPP)	4	-5.2	Mizushima y cols., 2004
Evolus® <sup>7</sup>	150 mL/día (2.25 mg IPP + 3-3.75 mg VPP)	8	-14.9	Seppo y cols., 2002
Evolus®	150 mL/día (2.25 mg IPP + 3-3.75 mg VPP)	21	-6.7	Seppo y cols., 2003
Evolus®	150 mL/día (2.4-2.7 mg IPP + 2.4-2.7 mg VPP)	10	-16	Tuomilehto y cols., 2004

(continúa en página siguiente)

**Tabla 3. (continuación)**

Producto	Dosis	Duración del tratamiento (semanas)	Efecto en la PAS <sup>1</sup> (mm Hg)	Referencias
Leche (enriquecida con un concentrado de proteínas del suero) fermentada por <i>Lactobacillus casei</i> TCMO409 y <i>Streptococcus thermophilus</i> TCM1543	2x200 mL/día	8	-7.0	Kawase y cols., 2000
Leche fermentada por <i>Lactobacillus helveticus</i> y por <i>Saccharomyces cerevisiae</i> conteniendo los péptidos IPP y VPP	95 mL/día	8	-12.1	Itakura y cols., 2001
Yogur con los tripéptidos IPP y VPP	300 mL/día	8	-13.9	Kajimoto y cols., 2002
Leche fermentada por <i>Lactobacillus helveticus</i> conteniendo los péptidos IPP y VPP	300 mL/día	10	-4.1	Jauhiainen y cols., 2005
Leche enriquecida con péptidos derivados de proteínas del suero	125mL/día	12	N.S. <sup>5</sup>	Lee y cols., 2007

<sup>1</sup> PAS: presión arterial sistólica.

<sup>2</sup> péptido aislado a partir de un hidrolizado enzimático de caseína.

<sup>3</sup> hidrolizado completo de caseína conteniendo los péptidos IPP y VPP.

<sup>4</sup> hidrolizado procedente de proteínas del suero de leche.

<sup>5</sup> no diferencias significativas respecto al control.

<sup>6</sup> leche fermentada por *Lactobacillus helveticus* y *Saccharomyces cerevisiae* conteniendo los péptidos IPP y VPP.

<sup>7</sup> leche fermentada por *Lactobacillus helveticus* LBK-16H conteniendo los péptidos IPP y VPP.

## **2.2. Diseño racional de nuevos péptidos antihipertensivos**

El desarrollo de la síntesis química de péptidos y sus análogos ha supuesto un gran avance en la caracterización e identificación de péptidos bioactivos, de forma que se pueden ensayar prácticamente a voluntad el efecto que tienen modificaciones, adiciones o deleciones concretas de determinados aminoácidos sobre la actividad de un péptido, o diseñar secuencias *de novo* y ensayar su actividad biológica.

Uno de los trabajos pioneros donde se estableció la importancia de la secuencia del dipéptido C-terminal en la unión de sustratos e inhibidores a la ECA, se basó en el diseño de amino terminal glicil-dipéptidos (Gly-X) y carboxilo terminal glicil-dipéptidos (X-Gly), y la determinación de su potencia inhibitoria (Cheung y cols., 1980). Esta aproximación permitió seleccionar los residuos triptófano, tirosina o prolina como los más efectivos para ocupar la posición C-terminal y los residuos valina, isoleucina y arginina para la posición amino terminal (N-terminal) de dipéptidos. De estas posibles combinaciones se describió como dipéptido más potente la secuencia VP ( $IC_{50} = 1.6 \mu M$ ), cuatro órdenes de magnitud más potente que el dipéptido con menor capacidad de inhibición, lo que demostró la alta especificidad que muestra la ECA con respecto al residuo dipeptídico C-terminal. Estos requerimientos de secuencia, conocidos como regla de Cheung, se mantienen en muchos de los péptidos descritos en la actualidad como inhibidores de la ECA, aunque la relación secuencia-función es mucho más compleja conforme aumenta la longitud de la cadena peptídica.

Otra estrategia en el diseño de nuevos péptidos bioactivos es la modificación de péptidos naturales para potenciar sus propiedades, dotándolos por ejemplo de mayor actividad o especificidad, o reduciendo aquellas no deseables como toxicidad inespecífica o poca estabilidad. Aunque esta estrategia se ha aplicado con éxito a la modificación de péptidos naturales, entre los que destacan aquellos con capacidad antimicrobiana (Marcos y cols., 2008), existen pocos ejemplos de

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modificación de péptidos antihipertensivos. En este contexto destaca el estudio llevado a cabo por Maruyama y cols. (1989) donde se modificó un péptido inhibidor de ECA de secuencia VHLPP ( $IC_{50} = 18 \mu M$ ) derivado de una proteína del endospermo del maíz. Basándose en las secuencias del extremo C-terminal de los primeros péptidos inhibidores de la ECA aislados a partir de veneno de serpiente (Ondetti y cols., 1971), se sustituyó el residuo de leucina por una isoleucina, o el penúltimo residuo de prolina del extremo C-terminal por un residuo de alanina. Estas modificaciones supusieron una mejora de la potencia inhibitoria *in vitro* (VHIPP,  $IC_{50} = 10 \mu M$ ; VHLAP,  $IC_{50} = 4.5 \mu M$ ), aunque no fue confirmada en ensayos con animales de experimentación. En un estudio llevado a cabo con péptidos derivados de caseína identificados en queso Manchego (Gómez-Ruiz y cols., 2004), se modificaron dos péptidos inhibidores de la ECA de secuencia VRYL ( $IC_{50} = 24.1 \mu M$ ) y KKYNVPL ( $IC_{50} = 77.1 \mu M$ ) con el fin de confirmar la importancia del residuo de leucina en el extremo C-terminal. Al sustituir dicho residuo por glicina, la potencia de los péptidos modificados se redujo 5 y 2 veces respectivamente (VRYG,  $IC_{50} = 132 \mu M$ ; KKYNVPLG,  $IC_{50} = 158.6 \mu M$ ). Asimismo, estos autores confirmaron la importancia del residuo en posición 4 desde el extremo C-terminal al determinar la potencia inhibitoria de los péptidos VRYL ( $IC_{50} = 24.1 \mu M$ ) y ERYL ( $IC_{50} = 200.3 \mu M$ ).

Desde mediados de los años noventa, la química combinatorial ha creado grandes expectativas en la identificación de nuevas sustancias bioactivas, debido a que posibilita la síntesis y ensayo de grandes colecciones de compuestos relacionados, denominadas bibliotecas de moléculas o quimiotecas (Blondelle y cols., 1996; Ganesan, 1998). Entre las quimiotecas combinatorias sintéticas más utilizadas destacan las compuestas por péptidos (peptidotecas). Las llamadas peptidotecas no definidas no presuponen ninguna restricción de secuencia y representan todas las combinaciones posibles de un n-péptido. Se han utilizado quimiotecas de hexapéptidos como compromiso entre un número de



mezclas manejable y una concentración efectiva en cada una de ellas (Blondelle y Houghten, 1996).

Por lo que se refiere a inhibidores de la ECA, se ha descrito la utilización de una peptidoteca compuesta por 228 dipéptidos para establecer las relaciones entre estructura, estabilidad, permeabilidad y capacidad de inhibición mediante un modelado QSAR (Foltz y cols., 2009). Este estudio permitió identificar los residuos aspártico, glicina y prolina en posición N-terminal y los residuos prolina, serina, treonina y aspártico en la posición C-terminal como factores de estabilidad aunque no fue posible establecer ninguna relación entre estructura y permeabilidad intestinal. Así mismo, se ha empleado una quimioteca de tripéptidos (373 secuencias) basada en la secuencia peptídica de la  $\alpha_{s1}$ -caseína bovina y la  $\beta$ -lactoglobulina para realizar un escrutinio de posibles secuencias inhibitoras de ECA (Ren y cols., 2011). Los tripéptidos más potentes correspondieron a secuencias derivadas de caseína, destacando por su potencia inhibitoria el péptido LKP ( $IC_{50} = 0.86 \mu M$ ).

En trabajos anteriores de nuestro grupo, se evaluaron los efectos inhibitorios sobre la ECA de una serie de siete hexapéptidos, previamente identificados como antifúngicos a partir de una peptidoteca (López-García y cols., 2002). Esta colección de hexapéptidos, caracterizada por compartir los residuos de aminoácidos arginina y lisina en su extremo N-terminal y fenilalanina y triptófano en el C-terminal, comparten la secuencia del dipéptido C-terminal con las secuencias aminoacídicas descritas como favorables para inhibir la ECA (Cheung y cols., 1980; Wu y cols., 2006). La evaluación de la serie de hexapéptidos de secuencia relacionada (Tabla 4) permitió seleccionar dos de ellos, PACEI32 (RKWHFW) y PACEI34 (RKWLFW) como inhibidores in vitro de la ECA, aunque sólo uno de ellos, PACEI32, mostró efecto inhibidor de la vasocontracción dependiente de ECA en ensayos funcionales ex vivo (Centeno y cols., 2006).

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Además, este estudio permitió señalar la importancia del tripéptido C-terminal, ya que los hexapéptidos con secuencias HFW y LFW en dicho extremo mostraron inhibición enzimática mientras que el hexapéptido con la secuencia RFW no fue capaz de inhibir la ECA.

**Tabla 4.** Secuencia de los hexapéptidos en los que se basa el presente trabajo [Adaptada de Centeno y cols., 2006].

Péptido	Secuencia <sup>a</sup>
P19	Ac-RK <b>T</b> W <b>F</b> W-NH <sub>2</sub>
P20	Ac-RK <b>T</b> P <b>F</b> W-NH <sub>2</sub>
P26	Ac-R <b>K</b> K <b>W</b> F <b>W</b> -NH <sub>2</sub>
P32	Ac-R <b>K</b> W <b>H</b> F <b>W</b> -NH <sub>2</sub>
P34	Ac-R <b>K</b> W <b>L</b> F <b>W</b> -NH <sub>2</sub>
P36	Ac-R <b>K</b> W <b>R</b> F <b>W</b> -NH <sub>2</sub>
P37	Ac-R <b>K</b> K <b>P</b> F <b>W</b> -NH <sub>2</sub>

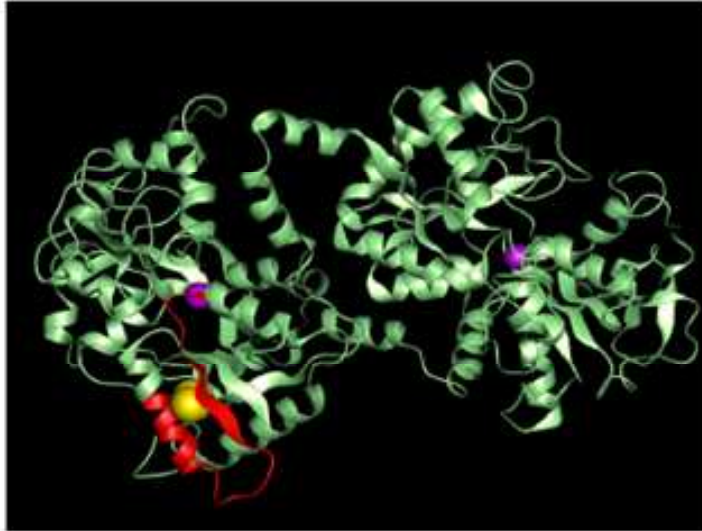
<sup>a</sup> Todos los péptidos están acetilados (Ac) en su extremo amino terminal y amidado (NH<sub>2</sub>) en su extremo carboxilo terminal. Los residuos de aminoácidos catiónicos R y K aparecen en rojo, los aromáticos W y F en azul.

### **2.3. Lactoferrina como fuente de péptidos bioactivos**

Las proteínas lácteas, y en especial las caseínas, son una de las fuentes principales de péptidos bioactivos y más concretamente de péptidos con actividad inhibidora de la ECA y efecto antihipertensivo (López-Fandiño y cols., 2006; Korhonen y Pihlanto, 2006; Ricci y cols., 2010). La  $\beta$ -lactoglobulina y la  $\alpha$ -lactoalbúmina, proteínas mayoritarias del suero lácteo, también han sido evaluadas como fuente de péptidos antihipertensivos (Pihlanto-Leppälä, 2001), pero existe poca información sobre el potencial de otros componentes proteicos como la lactoferrina (LF) que, aunque minoritaria, posee distintas propiedades biológicas (Tomita y cols., 2002; Orsi, 2004).

La LF es una glicoproteína quelante de hierro perteneciente a la familia de las transferrinas. Se localiza en superficies mucosas, en el interior de gránulos específicos de leucocitos polimorfonucleares, y también en fluidos biológicos tales como la leche, considerada la principal fuente de LF, la saliva y el fluido seminal, indicando que podría jugar un papel protector en la respuesta inmune innata (revisado en Farnaud y Evans, 2003).

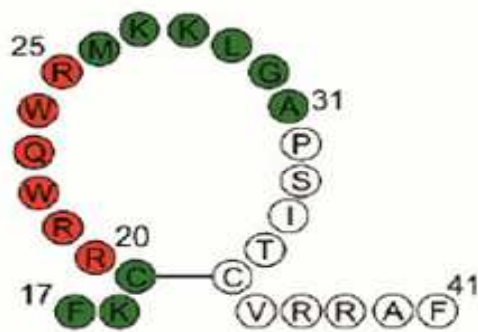
La LF es una glicoproteína monomérica bilobular con una masa molecular aproximada de 80 KDa. Sus dos lóbulos corresponden a los extremos N-terminal (residuos 1-333) y C-terminal (residuos 345-692) y son homólogos (37%), estando conectados por una cadena en  $\alpha$ -hélice (residuos 334-344) (Figura 3). Cada uno de estos lóbulos está subdividido en dos dominios, que poseen una zona de unión reversible a hierro. Cada átomo de hierro está coordinado a cuatro ligandos (dos residuos de tirosina, un aspartato y una histidina), además de a un anión, habitualmente carbonato (Williams, 1982; Metz-Boutigue y cols., 1984; Farnaud y Evans, 2003). Las LFs de origen humano y bovino poseen un 69% de homología de secuencia y una estructura terciaria muy similar (Pierce y cols., 1991).



**Figura 3.** Estructura tridimensional de la lactoferrina bovina. En rojo se señala la localización del fragmento amino terminal del cual se obtiene el péptido lactoferricin-B tras una hidrólisis con pepsina. Las bolas de color morado representan átomos de hierro y las amarillas puentes disulfuro.

Se ha descrito un amplio abanico de actividades para esta proteína, aunque en muchos casos su significación biológica no está clara. Entre ellas destacan las propiedades antimicrobianas y antivirales, acompañadas por las actividades antitumoral, antiinflamatoria, antioxidante e inmunomoduladora. Algunas de ellas están relacionadas con su capacidad para secuestrar hierro, mientras que otras parecen ser independientes de dicha propiedad (Tomita y cols., 2002; Orsi, 2004; Wakabayashi y cols., 2006; Weinberg, 2007).

Por lo que se refiere a péptidos derivados de LF, en un trabajo pionero sobre la estabilidad de proteínas antimicrobianas en el tracto digestivo, se demostró que mediante hidrólisis con pepsina de la LF bovina, se generaba un péptido, la lactoferricina bovina (LfcinB), que poseía mayor actividad antibacteriana que la proteína nativa (Bellamy y cols., 1992a; Bellamy y cols., 1992b). Este péptido, derivado de la región N-terminal del lóbulo N está constituida por 25 aminoácidos y comprende únicamente los residuos 17-41, tal y como se muestra en la Figura 4. Se ha descrito que tanto LfcinB como algunos péptidos derivados de su secuencia exhiben diversas actividades biológicas que además de la antibacteriana (Wakabayashi y cols., 1992; Branen y Davidson, 2000; Chen y cols., 2006) incluyen actividad antifúngica frente a diversas especies de levaduras (Van der Kraan y cols., 2005; Enrique y cols., 2007 y 2008) y hongos fitopatógenos (Muñoz y Marcos, 2006), así como propiedades antitumorales (Eliassen y cols., 2002 y 2003) y antivirales (Andersen y cols., 2004).



**Figura 4.** Secuencia del péptido LfcinB, mostrándose el puente disulfuro en su estructura. La numeración corresponde a la posición que ocupan los residuos en la proteína parental LFb. En rojo aparecen los aminoácidos del péptido LfcinB<sub>20-25</sub> y en verde los del péptido LfcinB<sub>17-31</sub> [adaptado de Bellamy y cols., 1992b]

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En un trabajo anterior de nuestro grupo, se examinó el efecto inhibitorio sobre la ECA de una serie de péptidos de secuencia relacionada con LfcinB (Centeno y cols., 2006). Entre ellos se evaluaron dos péptidos derivados de LfcinB, denominados LfcinB<sub>17-31</sub> (FKCRRWQWRMKKLG) y LfcinB<sub>20-25</sub> (RRWQWR) (ver Figura 4). Ambos péptidos inhibieron la ECA en ensayos in vitro y la evaluación del primero de ellos en ensayos funcionales con arterias aisladas permitió demostrar su efecto inhibitorio sobre la contracción dependiente de ECA. Coincidiendo en el tiempo con este trabajo, Lee y cols. (2006) describieron un hexapéptido derivado de LF con efecto antihipertensivo en ratas SHR tras administración intravenosa. Este péptido, de secuencia LRPVAA, derivado de una zona de LF diferente a la zona de la cual deriva LfcinB, fue aislado a partir de un hidrolizado de LF obtenido con pepsina. Ambos trabajos son las dos únicas publicaciones donde se caracteriza parcialmente el efecto antihipertensivo de péptidos derivados de LF, a pesar de que previamente un estudio de análisis bioinformático llevado a cabo por Vermeirssen y cols. (2004) la señalaba como la proteína del suero lácteo con mayor potencial para generar péptidos con efectos inhibitorios sobre la ECA.

## **OBJETIVOS**

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El objetivo del presente trabajo es la obtención de péptidos con efecto antihipertensivo. Para ello se evaluará el potencial antihipertensivo de péptidos derivados de lactoferrina bovina (LFb), completando la caracterización de péptidos derivados de la lactoferrina bovina (LfcinB) iniciada por nuestro grupo, y se identificarán nuevos péptidos derivados de otras localizaciones de la secuencia de la proteína. Asimismo, y basándonos en los resultados previos del grupo con hexapéptidos no naturales, se pretende, mediante diseño racional, identificar heptapéptidos con efecto antihipertensivo mejorado.

Teniendo en cuenta el objetivo general expuesto, se proponen los siguientes objetivos parciales:

**OBJETIVO 1.**

Caracterizar el efecto antihipertensivo de péptidos derivados de LfcinB y evaluar las posibles relaciones secuencia-efecto antihipertensivo. Para ello se emplearán péptidos derivados de diferentes elongaciones de los extremos C-terminal y N-terminal de LfcinB<sub>20-25</sub> y LfcinB<sub>17-31</sub>, se evaluará su capacidad para inhibir la actividad de ECA *in vitro* y la vasoconstricción ECA-dependiente *ex vivo*, y se estudiará su efecto antihipertensivo en ratas espontáneamente hipertensas (SHR). Asimismo se incluirán estudios sobre la estabilidad gastrointestinal de los péptidos y la funcionalidad de los posibles fragmentos resultantes.

**OBJETIVO 2.**

Evaluar el potencial de LFb como fuente de péptidos antihipertensivos procedentes de zonas diferentes a LfcinB e identificar los péptidos responsables del efecto antihipertensivo. Para ello se obtendrán hidrolizados de LFb utilizando pepsina como enzima hidrolítico y, demostrado su potencial antihipertensor, se identificarán mediante espectrometría de masas en tándem (RP-HPLC-MS/MS) los péptidos presentes en el hidrolizado y/o fracciones del mismo que pudieran ser

## Objetivos

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responsables de dicho efecto. La caracterización de los hidrolizados y péptidos incluirá la capacidad para inhibir la actividad ECA y la vasoconstricción ECA-dependiente, así como el efecto antihipertensivo en SHR.

### **OBJETIVO 3.**

Diseñar heptapéptidos con efecto antihipertensivo a partir de hexapéptidos con demostrada capacidad inhibidora de ECA. Para ello, se comprobará su capacidad para inhibir la actividad ECA y la vasoconstricción ECA-dependiente y se estudiará el efecto antihipertensivo en SHR de los heptapéptidos y los hexapéptidos parentales. Se comprobará la estereoselectividad en los efectos de los péptidos, y dado que se trata de péptidos no naturales se estudiarán los posibles efectos citotóxicos de los péptidos con efecto antihipertensor.

## **ARTÍCULOS ORIGINALES**



Esta tesis es una compilación de los cuatro artículos originales publicados que se indican a continuación:

- I. Antihypertensive properties of lactoferricin B-derived peptides. **Ruiz-Giménez, P.**, Ibañez, A., Salom, J.B., Marcos, J.F., López-Díez, J.J., Vallés, S., Torregrosa, G., Alborch, E. and Manzanares, P. *Journal of Agricultural and Food Chemistry*, 58, 6721-6727, 2010.
- II. Bovine lactoferrin pepsin hydrolysate exerts inhibitory effect on angiotensin I-converting enzyme-dependent vasoconstriction. **Ruiz-Giménez, P.**, Burguete, M.C., Castelló-Ruiz, M., Marcos, J.F., Salom, J.B., Vallés, S., Torregrosa, G., Alborch, E. and Manzanares, P. *International Dairy Journal*, 17, 1212-1215, 2007.
- III. Antihypertensive effect of a bovine lactoferrin pepsin hydrolysate: Identification of a novel active peptides. **Ruiz-Giménez, P.**, Salom, J.B., Marcos, J.F., Vallés, S., Martínez-Maqueda, D., Recio, I., Torregrosa, G., Alborch, E. and Manzanares, P. *Food Chemistry*, 131, 266-273, 2012.
- IV. Novel antihypertensive hexa- and heptapeptides with ACE-inhibiting properties: From the in vitro ACE assay to the spontaneously hypertensive rat. **Ruiz-Giménez, P.**, Marcos, J.F., Torregrosa, G., Lahoz, A., Fernández-Musoles, R., Vallés, S., Alborch, E., Manzanares, P. and Salom, J.B. *Peptides*, 32, 1431-1438, 2011.



Artículo I

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**Antihypertensive Properties of Lactoferricin B-Derived Peptides**





**ABSTRACT**

A set of eight lactoferricin B (LfcinB)-derived peptides was examined for their inhibitory effects on angiotensin I-converting enzyme (ACE) activity and ACE-dependent vasoconstriction, and their hypotensive effect in spontaneously hypertensive rats (SHR). Peptides derived from different elongations both at the C-terminal and N-terminal end of the representative peptide LfcinB<sub>20-25</sub>, which is known as the LfcinB antimicrobial core. All the eight LfcinB-derived peptides showed *in vitro* inhibitory effects on ACE activity with different IC<sub>50</sub> 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<sub>20-25</sub> 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<sub>20-25</sub>. In conclusion, two LfcinB-derived peptides lower blood pressure and exhibit potential as orally effective antihypertensive compounds, yet 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.

## 1. INTRODUCTION

In recent years, interest on 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 humans (1, 2). This biological function seems to be related to 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).

Most of 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 the 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 have been deeply studied (11), as well as whey protein derived peptides (12). 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, 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<sub>17-31</sub> (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 end of LfcinB<sub>20-25</sub> (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<sub>20-25</sub> fragments obtained after simulated digestion were also carried out.

## 2. 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  $-20^{\circ}\text{C}$ . Peptide concentrations were determined by measuring the absorbance at 280 nm ( $\epsilon^{280} = 5600 \text{ M}^{-1} \text{ cm}^{-1}$  for W residue).

Fragments derived from LfcinB<sub>20-25</sub> 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 peptide inhibitory effect on ACE was expressed by using the  $\text{IC}_{50}$ , 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).

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

Peptide <sup>a</sup>	Sequence
LfcinB <sub>17-32</sub>	Ac-FKCRRWQWRMKKLGAP-NH <sub>2</sub>
LfcinB <sub>17-31</sub>	Ac-FKCRRWQWRMKKLG-NH <sub>2</sub>
LfcinB <sub>20-25</sub>	Ac-RRWQWR-NH <sub>2</sub>
LfcinB <sub>19-25</sub>	Ac-CRRWQWR-NH <sub>2</sub>
LfcinB <sub>18-25</sub>	Ac-KCRRWQWR-NH <sub>2</sub>
LfcinB <sub>17-25</sub>	Ac-FKCRRWQWR-NH <sub>2</sub>
LfcinB <sub>17-24</sub>	Ac-FKCRRWQW-NH <sub>2</sub>
LfcinB <sub>17-22</sub>	Ac-FKCRRW-NH <sub>2</sub>
LfcinB <sub>22-23</sub> -F1	WQ
LfcinB <sub>21-23</sub> -F2	RWQ

<sup>a</sup>All of the peptides were acetylated at the N-terminus (Ac) and amidated at the C-terminus (NH<sub>2</sub>) with the exception of LfcinB<sub>22-23</sub>-F1 and LfcinB<sub>21-23</sub>-F2.

**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. Due to stress associated with transportation, at least 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 killed by injection of 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<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, 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 non-invasive tail-cuff method (21) using a 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 hours 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 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 analyze by RP-HPLC each treatment independently, as specified above. Each sample was stored at -20°C until further analysis.

### 3. RESULTS

***In Vitro* ACE-Inhibitory Activity of LfcinB-Derived Peptides.** In a previous work, we found no significant difference in *in vitro* ACE inhibitory activity between LfcinB<sub>20-25</sub> and LfcinB<sub>17-31</sub>, tested at a concentration of 20  $\mu$ M, suggesting that the elongation of the LfcinB<sub>20-25</sub> 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 end of Lfcin<sub>20-25</sub> (Table 1). Table 2 shows the ACE inhibitory activity of peptides. Confirming our previous results, no differences in IC<sub>50</sub> values were found between LfcinB<sub>20-25</sub> and LfcinB<sub>17-31</sub>. Interestingly different elongations of LfcinB<sub>20-25</sub> provoked differences in inhibitory potency. The higher potency as indicated by lower IC<sub>50</sub> values corresponded to peptides LfcinB<sub>19-25</sub>, LfcinB<sub>17-25</sub> and LfcinB<sub>18-25</sub>, derived from elongations at the LfcinB<sub>20-25</sub> N-terminal end. On the other hand the IC<sub>50</sub> values for peptides LfcinB<sub>17-25</sub>, LfcinB<sub>17-24</sub> and LfcinB<sub>17-22</sub> 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<sub>17-31</sub> provoked a two-fold increase in the inhibitory potency of resultant LfcinB<sub>17-32</sub>.

***Ex Vivo* Inhibitory Effect of LfcinB-Derived Peptides on ACE-Dependent Vasoconstriction.** We have previously reported the inhibitory effect of LfcinB<sub>17-31</sub> (20  $\mu$ M) on angiotensin I-induced contraction of the rabbit carotid artery (control: 86  $\pm$  3 %, n = 27; LfcinB<sub>17-31</sub>: 68  $\pm$  6 %, n = 10) (16). In the present study, challenge of the arterial segments with depolarizing solution (KCl 50 mM) induced contraction averaging 3862  $\pm$  136 mg (n = 88). Angiotensin I (1  $\mu$ M) induced phasic, transient contractions almost completely reproducible (89  $\pm$  3 %, n=19) in two consecutive challenges to the same arterial segment. Preincubation (20 min) with LfcinB-derived peptides (20  $\mu$ M) induced different inhibitory effects on angiotensin I-induced contractions (representative recordings are



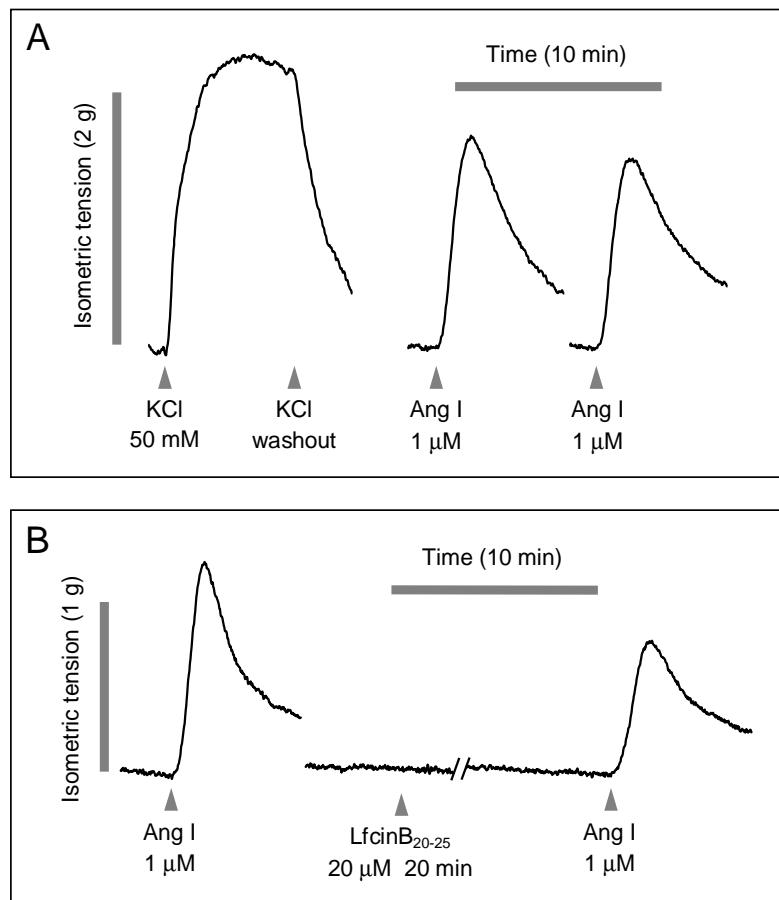
shown in Figure 1). As summarized in Table 3, all of the peptides tested, with the exception of LfcinB<sub>19-25</sub>, induced significant inhibitions when compared to the control. As a general trend, there was no correlation between the *in vitro* potency of LfcinB-derivatives and their effect on ACE-dependent vasoconstriction (compare Tables 2 and 3).

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

Peptide	IC <sub>50</sub> (μM) <sup>a</sup>
LfcinB <sub>17-32</sub>	11.0 ± 1.5 (4) b
LfcinB <sub>17-31</sub> <sup>b</sup>	25.5 ± 2.3 (4) c
LfcinB <sub>20-25</sub>	32.0 ± 4.9 (3) c
LfcinB <sub>19-25</sub>	2.3 ± 0.1 (3) a
LfcinB <sub>18-25</sub>	5.8 ± 0.2 (3) a, b
LfcinB <sub>17-25</sub>	2.9 ± 0.6 (7) a
LfcinB <sub>17-24</sub>	10.5 ± 0.6 (3) b
LfcinB <sub>17-22</sub>	26.7 ± 1.9 (4) c

<sup>a</sup> Inhibitory potency is expressed as IC<sub>50</sub>, and data are 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-Keuls tests.

<sup>b</sup> Ref 16.



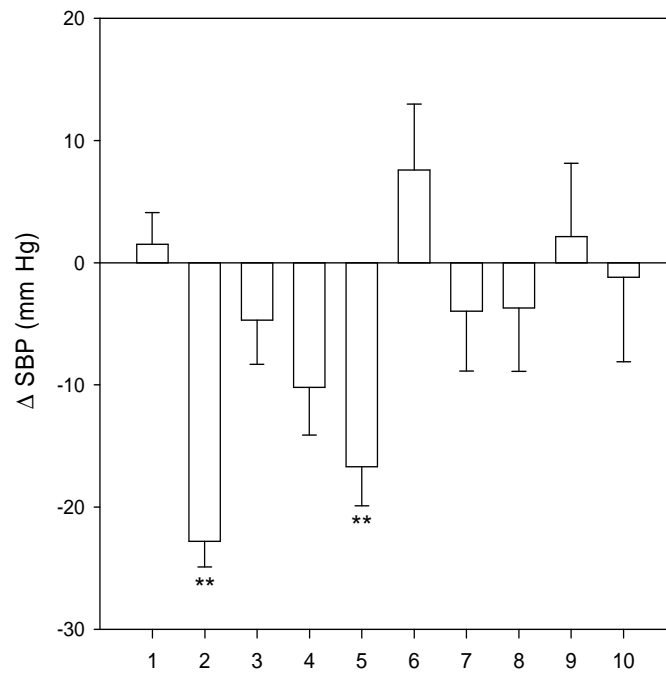
**Figure 1.** Contraction of carotid artery segments. **(A)** Effect of high-KCl to check for tissue viability and reproducible vasoconstriction to angiotensin I. **(B)** Effect of LfcinB<sub>20-25</sub> on angiotensin I-induced contraction.

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

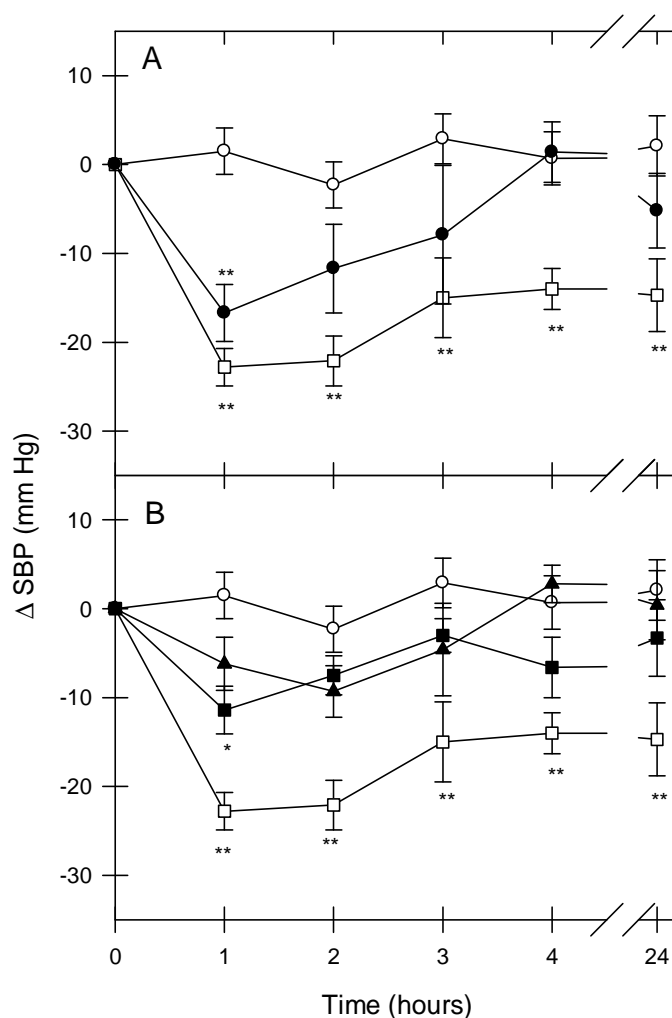
Peptide <sup>a</sup>	Contraction peak (%) <sup>b</sup>
Control	89 ± 3 (19)
LfcinB <sub>17-32</sub>	71 ± 2 (6) <sup>d</sup>
LfcinB <sub>20-25</sub>	63 ± 3 (5) <sup>d</sup>
LfcinB <sub>19-25</sub>	86 ± 3 (7)
LfcinB <sub>18-25</sub>	67 ± 3 (6) <sup>d</sup>
LfcinB <sub>17-25</sub>	66 ± 2 (10) <sup>d</sup>
LfcinB <sub>17-24</sub>	73 ± 4 (6) <sup>c</sup>
LfcinB <sub>17-22</sub>	64 ± 1 (7) <sup>d</sup>

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

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



**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  $\pm$  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<sub>17-32</sub>; 4, LfcinB<sub>17-31</sub>; 5, LfcinB<sub>20-25</sub>; 6, LfcinB<sub>19-25</sub>; 7, LfcinB<sub>18-25</sub>; 8, LfcinB<sub>17-25</sub>; 9, LfcinB<sub>17-24</sub>; 10, LfcinB<sub>17-22</sub>.



**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<sub>20-25</sub> (Ac-RRWQWR-NH<sub>2</sub>); **Panel B:** (■), LfcinB<sub>22-23</sub>-F1 (WQ); (▲), LfcinB<sub>21-23</sub>-F2 (RWQ). Pressure changes are expressed in absolute values (mm Hg) and data are expressed as mean  $\pm$  SEM for a minimum of four animals (n = 4-6), \* $P$ <0.05 versus control group, \*\* $P$ <0.01 versus control group (one-way ANOVA followed by Dunnett's multiple comparison tests).

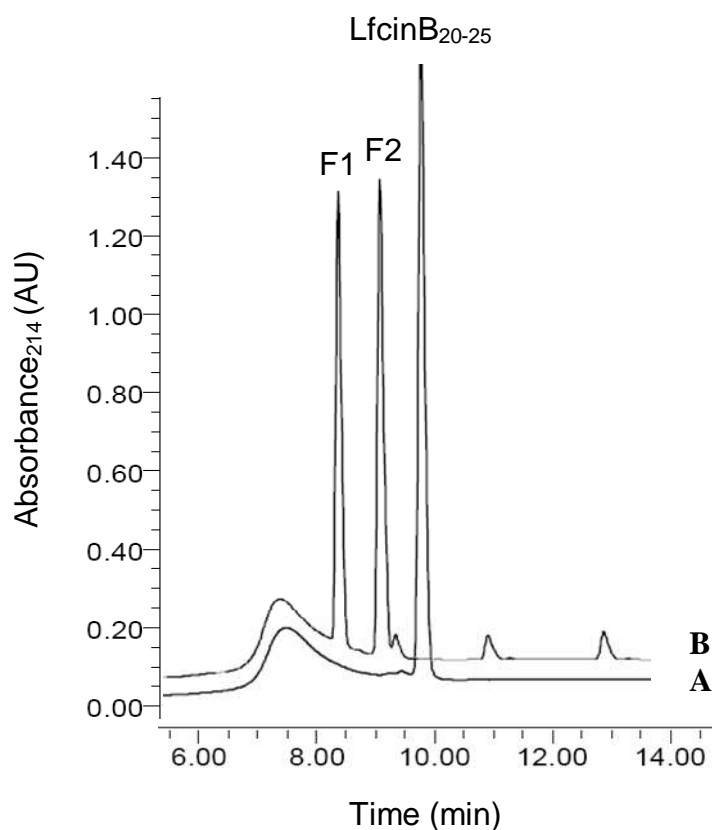
**Simulated Gastrointestinal Digestion and Intestinal Stability of LfcinB-Derived Peptides.** In order to investigate the cause(s) for the lack of *in vivo* antihypertensive effect of most of the LfcinB-derived peptides, they were subjected to a hydrolysis process which simulates physiological digestion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

The *in vitro* ACE inhibitory activities of the resulting digests were firstly determined. Most of the peptides showed  $IC_{50}$  values higher than 100  $\mu M$  after digestion. Only digests of LfcinB<sub>17-31</sub>, LfcinB<sub>19-25</sub> and LfcinB<sub>17-24</sub> showed  $IC_{50}$  values of  $20.5 \pm 5.4$ ,  $5.3 \pm 0.4$  and  $18.7 \pm 2.5$   $\mu M$  ( $n = 3$ ), 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<sub>17-31</sub>, LfcinB<sub>19-25</sub> and LfcinB<sub>17-24</sub> were only partly hydrolyzed (approximately 50 % of the initial concentration of the input peptide) whereas the rest of LfcinB derivatives were almost completely hydrolyzed (hydrolysis > 90%). No significant differences between peptide profiles after Corolase stage and intestinal peptidase treatment were observed.

**Identification and Functional Characterization of LfcinB<sub>20-25</sub> Fragments Released by Simulated Digestion.** Although the LfcinB<sub>20-25</sub> digest had no significant *in vitro* activity ( $IC_{50} > 100$   $\mu M$ ), this peptide showed hypotensive effect *in vivo* (Figure 3) and was completely hydrolysed 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  $\mu M$ ) showed significant effect on ACE-dependent, angiotensin I-induced vasoconstriction (control:  $93 \pm 3$  %,  $n = 8$ ; F1:  $82 \pm 3$  %,  $n = 8$ ; F2:  $83 \pm 5$

%,  $n = 6$ ;  $P > 0.05$ , 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 ( $-11.4 \pm 2.7$  mm Hg) whereas the slight decrease in SBP caused by F2 was not significant.



**Figure 4.** RP-HPLC chromatograms of LfcinB<sub>20-25</sub> before (A) and after (B) being submitted to simulated gastrointestinal digestion. LfcinB<sub>22-23</sub>-F1 (WQ) and LfcinB<sub>21-23</sub>-F2 (RWQ) were identified by RP-HPLC-MS/MS.

#### 4. DISCUSSION

In a previous work we showed the vasoactive effects of the antimicrobial LfcinB-derived peptide LfcinB<sub>17-31</sub> through ACE inhibition (16). In this study we further characterized the antihypertensive activity of LfcinB<sub>17-31</sub> and of a set of LfcinB-derived peptides obtained from different N-terminal and C-terminal elongations of LfcinB<sub>20-25</sub>, 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<sub>50</sub> 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<sub>19-25</sub>, LfcinB<sub>18-25</sub> and LfcinB<sub>17-25</sub>, derived from elongations at the N-terminal end of LfcinB<sub>20-25</sub> showed higher *in vitro* potency than the parental one. All of them have R as the C-terminal residue which positive charge has been described to contribute substantially 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, F) and ultimate position (W, Y, F, P, I, A, L, M) of ACE inhibitors and substrates. The different ACE inhibitory potency found between LfcinB<sub>17-25</sub> and LfcinB<sub>17-24</sub> reinforces the importance of the R residue at the C-terminal position, whereas the differences between LfcinB<sub>17-31</sub> and LfcinB<sub>17-32</sub> support the role of 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 rabbit isolated 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<sub>19-25</sub> with a high *in vitro* inhibitory potency (IC<sub>50</sub> 2.3 ± 0.2 μM) did not show any effect on ACE-dependent vasoconstriction in contrast to LfcinB<sub>20-25</sub>, LfcinB<sub>17-31</sub> and LfcinB<sub>17-22</sub> that had *in vitro* IC<sub>50</sub> 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 ACE substrate in both experiments. In our previous study other LfcinB-related peptides with *in vitro* ACE inhibitory activity did not show *ex vivo* effect (16). In the context of the peptides studied, the addition of a C residue to the N-terminal end of LfcinB<sub>20-25</sub> provoked the loss of LfcinB<sub>19-25</sub> inhibitory activity although the successive addition of K (LfcinB<sub>18-25</sub>) and F (LfcinB<sub>17-25</sub>) 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<sub>17-31</sub> 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<sub>20-25</sub> showed a moderate hypotensive effect (-16.7 ± 3.2 mm Hg) 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 mm Hg 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<sub>20-25</sub> 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 ( $-11.4 \pm 2.7$  mm Hg) in SHR, that is 5.8 % reduction from baseline SBP, lower than that caused by LfcinB<sub>20-25</sub>. 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<sub>20-25</sub> may be responsible of the *in vivo* effect. The possibility of a synergistic effect among LfcinB<sub>20-25</sub> and its fragments can not 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 blood stream (32). In some cases the utility of simulated gastrointestinal digestion and serum incubation as a screening protocol for 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 human stomach (33). Also two peptides derived from a different lactoferrin region [f(382-389) and f(442-447)] were identified in human intestine 20 min after milk ingestion (34).

In contrast to that found for LfcinB<sub>20-25</sub>, the hypotensive fragment WQ did not show any effect on ACE-dependent vasoconstriction. This result agreed with the *in vitro* value of IC<sub>50</sub> (>100 μM) determined for the LfcinB<sub>20-25</sub> 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 the argininosuccinate synthetase, an important player of 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<sub>20-25</sub> is the first lactoferrin-derived peptide described as having 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<sub>20-25</sub>, 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<sub>20-25</sub> 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.

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Artículo II

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**Bovine lactoferrin pepsin hydrolysate exerts inhibitory effect on angiotensin I-converting enzyme-dependent vasoconstriction**



**ABSTRACT**

The inhibitory effect of a pepsin hydrolysate of bovine lactoferrin (LFH) on angiotensin I-converting enzyme (ACE) has been examined using in vitro and ex vivo functional assays. In vitro assays showed LFH inhibitory effect on ACE activity with an  $IC_{50}$  value of  $0.95 \pm 0.06$  mg mL<sup>-1</sup>. Ex vivo functional assays using rabbit carotid artery segments showed LFH inhibitory effect on ACE-dependent angiotensin I-induced contraction but not on angiotensin II-induced contraction, suggesting that the effect of LFH is not due to antagonism of receptors for angiotensin II. In conclusion, LFH possesses ACE inhibitory effect with potential to modulate hypertension, although the possible inhibitory effect of LFH on angiotensinases deserves further research.

**Keywords:** Bovine lactoferrin; Pepsin hydrolysate; ACE inhibition; ACE-dependent vasoconstriction; Hypertension.

## **1. INTRODUCTION**

Lactoferrin (LF), a major constituent of mammal milk, is a multifunctional iron glycoprotein which is known to exhibit a diverse range of biological effects, including antimicrobial, antiviral, antioxidant, and immunomodulatory activities (Tomita, Wakabayashi, Yamauchi, Teraguchi & Hayasawa, 2002). It is now accepted that enzymatic hydrolysis of food protein releases peptides that may exhibit different or enhanced biological activities. In this context, pepsin digested LF has been found more effective than LF as antimicrobial against several bacteria, showing a bactericidal effect instead of the bacteriostatic effect of LF (Tomita, Bellamy, Takase, Yamauchi, Wakabayashi & Kawase, 1991). The active peptides responsible for the antimicrobial activity of the LF hydrolysate were subsequently identified (Bellamy, Takase, Yamauchi, Wakabayashi, Kawase & Tomita, 1992) and were named lactoferricin H from human milk (LfcinH) and lactoferricin B from bovine milk (LfcinB). Moreover, two additional antibacterial peptides different to Lfcin have been isolated from a pepsin digest of bovine LF (Dionysius & Milne, 1997).

The renin-angiotensin system (RAS) is a coordinated peptidic hormonal cascade for the control of cardiovascular, renal and adrenal functions governing fluid and electrolyte balance and arterial blood pressure (Carey & Siragy, 2003). Briefly, prorenin is converted to active renin by a trypsin-like enzyme. Renin cleaves angiotensinogen to form angiotensin I. Angiotensin I-converting enzyme (ACE) hydrolyzes both the inactive angiotensin I into vasoconstrictor angiotensin II and the vasodilator bradikinin into an inactive metabolite. Most well known effects of angiotensin II, including vasoconstriction, are mediated by angiotensin type 1 receptors (AT<sub>1</sub>). Angiotensin II also binds to angiotensin type 2 receptors (AT<sub>2</sub>) which are highly expressed in fetal mesenchymal tissues but poorly expressed in the adult.

Among peptides with biological functions, interest in identifying ACE inhibitors has grown due to their beneficial effects in the treatment of hypertension and heart failure. Many studies have been performed on antihypertensive peptides derived from milk proteins, mainly caseins (for reviews see FitzGerald, Murray & Walsh, 2004; Korhonen & Pihlanto, 2006). Recently we have demonstrated inhibitory effects on ACE activity and ACE-dependent vasoconstriction of synthetic Lfcin-related peptides including two representative LfcinB-derived peptides, LfcinB<sub>20-25</sub> and LfcinB<sub>17-31</sub>. We used an ex vivo model, the rabbit isolated carotid artery, in which angiotensin I induces ACE-dependent vasoconstriction checked by inhibition with the selective inhibitor captopril (Centeno et al., 2006). Local formation of angiotensin II from angiotensin I by ACE present in the arterial wall is necessary for induction of contraction by inactive angiotensin I (Egleme, Cressier & Wood, 1990).

However the synthetic procedure used for Lfcin-related peptide production hampers their potential practical use. Moreover these peptides acetylated at the N-terminus and amidated at the C-terminus may not be representative of the bioactivity present in peptides naturally produced on LF incubation with pepsin. These facts point to the suitability for examining the ACE inhibitory activity of bovine LF and derived hydrolysates. Thus, the aim of the present study was to evaluate the ACE inhibitory effect of bovine LF and a bovine LF pepsin hydrolysate (LFH) using both in vitro and ex vivo functional assays.

## **2. MATERIALS AND METHODS**

### **2.1. Lactoferrin hydrolysis**

Bovine LF (USB Corp., Cleveland, Ohio, USA) was dissolved to 50 mg mL<sup>-1</sup> and hydrolysed using porcine pepsin (3% w/w; Sigma Chemical Co., St. Louis, MO, USA) according to the procedure previously described (Tomita et al., 1991). The Bradford procedure using bovine serum albumin as standard (Bradford, 1976) was used to estimate protein concentration. The LFH protein concentration was taken to be equal to the protein content of the original (non-hydrolysed) LF solution.

### **2.2. In vitro assay of ACE inhibitory effect**

LFH was assayed in vitro for its capacity to inhibit the ACE activity using hippuryl-L-histidyl-L-leucine (HHL; Sigma) as substrate and following the protocol described previously (Centeno et al., 2006). ACE (from porcine kidney) was purchased from Sigma.

Potency of the ACE inhibitory effect was expressed as the protein concentration required to inhibit 50% of the ACE activity without inhibitor (IC<sub>50</sub>). A non-linear regression of the experimental data to a four-parameter logistic curve using the software package SigmaPlot v 8.02 (SPSS Inc., Chicago, IL, USA) was performed to calculate the IC<sub>50</sub>.

### **2.3. 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. Eighteen male New Zealand White rabbits (Technology Transferring Center, Polytechnic University of Valencia, Spain), weighing 2.5-3 kg, were killed by injection of 25 mg kg<sup>-1</sup> sodium thiopental (Tiobarbital, 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 the common carotid artery, which was dissected free and cut in four 4-mm long segments.



**2.4. Ex vivo functional assay of ACE inhibitory effect**

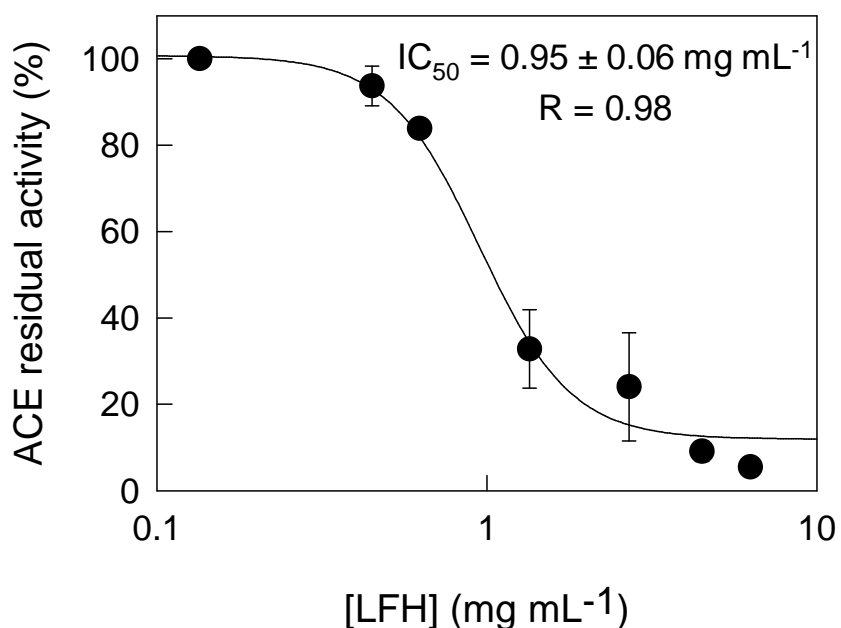
For isometric tension recording, the arterial segments were mounted in an organ bath by using tungsten wires (207  $\mu\text{m}$  in diameter). Two pins were introduced through the arterial lumen: one pin was fixed to a stationary support, while the other one was connected to a strain gauge (Universal Transducing Cell UC3, Gould Statham, Oxnard, CA, USA). Isometric tension was conveniently amplified (OCTAL Bridge, ADInstruments, Castle Hill, Australia), digitized (PowerLab/8SP, ADInstruments), recorded and stored in an IBM<sup>®</sup> PC compatible 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  $\text{CaCl}_2$ , 1.0 mM  $\text{MgCl}_2$ , 25 mM  $\text{NaHCO}_3$  and 5.6 mM glucose) at 37°C and bubbled with a 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  mixture to give a pH of 7.3-7.4. 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 starting the experiments.

The contractile capacity of every arterial segment was assessed by exposure to 50 mM KCl Ringer-Locke solution (NaCl was replaced by 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  $\mu\text{M}$ ) 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, preincubation with increasing concentrations of LFH (0.45, 1.35 or 4.49  $\text{mg mL}^{-1}$ ) and a second challenge to angiotensin I to check for their effect on angiotensin I-induced contraction, preincubation with native LF (4.49  $\text{mg mL}^{-1}$ ) or pepsin (1.5  $\text{mg mL}^{-1}$ ) and a second challenge to angiotensin I as negative controls. Additionally, some arterial segments were subjected to the first and second protocols but with angiotensin II instead of angiotensin I in order to discard effects of LFH on angiotensin receptors.

Two parameters feature contraction responses: contraction peak and time spent to reach this peak. The second contraction peak to angiotensin I or angiotensin II is expressed as percentage of the first contraction in the same arterial segment. Time is expressed in absolute values (s). All data are expressed as mean  $\pm$  SEM from 'n' arterial segments obtained from different rabbits. Unpaired t-test or one-way ANOVA followed by Dunnett multiple comparison test were used to compare the control and experimental groups (GraphPad InStat 3.06, GraphPad Software Inc, San Diego, CA, USA).  $P < 0.05$  was considered significant.

### 3. RESULTS AND DISCUSSION

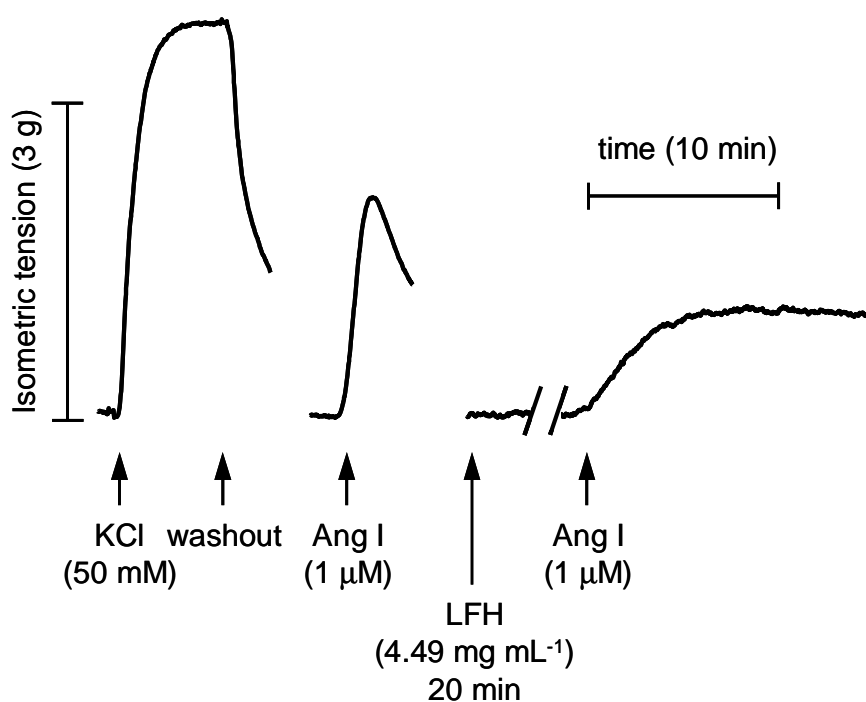
In preliminary in vitro experiments, LFH incubation resulted in ACE residual activity of  $9 \pm 2\%$  ( $n=8$ ) when tested at a concentration of  $4.49 \text{ mg mL}^{-1}$ . In parallel experiments, pepsin ( $1.5 \text{ mg mL}^{-1}$ ) and non-hydrolysed LF ( $4.49 \text{ mg mL}^{-1}$ ) did not show any inhibitory effect on ACE activity suggesting that LF-derived peptides by pepsin hydrolysis possess the inhibitory activity. Figure 1 shows a concentration-response curve on the effect of LFH on ACE residual activity. LFH exhibited an  $\text{IC}_{50}$  value of  $0.95 \pm 0.06 \text{ mg mL}^{-1}$ .



**Figure 1.** Effect of LFH concentration on ACE residual activity. Data are the mean  $\pm$  SEM of at least three independent experiments.

The ex vivo assay using rabbit isolated carotid arteries is a suitable system for the functional evaluation of ACE inhibition in vascular tissue. We have previously reported that angiotensin I induces ACE-dependent vasoconstriction 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 (Centeno et al., 2006). In the present study, challenge of rabbit carotid arterial segments with depolarising solution (KCl 50 mM) induced phasic contraction followed by active tone maintained until KCl washout (Figure 2). Average active tone amounted to  $4456 \pm 148$  mg ( $n=63$ ). Angiotensin I ( $1 \mu\text{M}$ ) or angiotensin II ( $1 \mu\text{M}$ ) induced phasic, transient contractions almost completely reproducible (more than 80%) in two consecutive challenges to the same arterial segment. Preincubation (20 min) with LFH flattened angiotensin I-induced contraction by both reducing contraction peak and delaying time to peak (a representative recording is shown at Figure 2). Table 1 summarizes concentration-dependent inhibitory effects of LFH on angiotensin I-induced contraction. As can be seen LFH preincubation ( $4.49 \text{ mg mL}^{-1}$  and  $1.35 \text{ mg mL}^{-1}$ ) induced significant inhibitions when compared to the control. Although contractions to angiotensin I in arterial segments preincubated with LFH at a lower concentration of  $0.45 \text{ mg mL}^{-1}$  were weaker than the control response, the reduction did not reach significance. Non-hydrolysed LF and pepsin did not show any effect on ACE-dependent vasoconstriction. On the other hand, preincubation with LFH ( $4.49 \text{ mg mL}^{-1}$ ) did not inhibit angiotensin II-induced contraction suggesting that the inhibitory effect of LFH is not due to antagonism of  $\text{AT}_1$  receptors, which mediate vasoconstriction induced by angiotensin II (Carey & Siragy, 2003). A significant potentiation of contraction peak to angiotensin II was induced instead (control  $82 \pm 3\%$  vs. LFH  $108 \pm 11\%$ ,  $P<0.05$ ,  $n=5$ ). Angiotensin II is degraded within seconds by peptidases, collectively termed angiotensinases, at different amino acid sites to form inactive fragments like des-aspartyl-angiotensin II (angiotensin III), angiotensin (1-7) and angiotensin (3-8) (Carey & Siragy, 2003). The potentiation of angiotensin II-

induced contraction suggests that LFH, in addition to the inhibitory effect on ACE, may also have some inhibitory effect on subsequent degradation of vasoconstrictor angiotensin II yielding non vasoactive peptides.



**Figure 2.** Representative recording of contraction responses in rabbit carotid artery. Effect of KCl to check for tissue viability and inhibitory effect of LFH preincubation ( $4.49 \text{ mg mL}^{-1}$ , 20 min) on angiotensin I-induced contraction.

**Table 1.** Concentration-dependent effects of LFH<sup>a</sup> on angiotensin I-induced contraction<sup>b</sup>

	Contraction Peak (%) <sup>c</sup>	Time to Peak (s) <sup>d</sup>	n
Control	83 ± 3	104 ± 4	18
LFH, 0.45 mg mL <sup>-1</sup>	69 ± 6	191 ± 8	8
LFH, 1.35 mg mL <sup>-1</sup>	60 ± 6 *	263 ± 32	6
LFH, 4.49 mg mL <sup>-1</sup>	44 ± 5 *	695 ± 118 *	11
LF, 4.49 mg mL <sup>-1</sup>	77 ± 9	126 ± 12	5
Pepsin, 1.5 mg mL <sup>-1</sup>	78 ± 6	125 ± 19	5

<sup>a</sup>LFH: lactoferrin pepsin hydrolysate. LF: lactoferrin.

<sup>b</sup>Data are expressed as mean ± SEM from `n´ arterial segments.

<sup>c</sup>Second contraction response to angiotensin I expressed as percentage of the first contraction in the same arterial segment.

<sup>d</sup>Time spent to reach maximal contraction.

\*An asterisk indicates significantly different from control,  $P < 0.01$ .

The formation of antihypertensive peptides by milk protein enzymatic hydrolysis and milk fermentation has been extensively studied, but only few among them have so far proven effective in animal and human studies (Meisel, 2005, Korhonen & Pihlanto, 2006). LF-derived peptides, initially characterised as having antimicrobial activity, also possess antiviral, antitumor and immunological properties (Gifford, Hunter & Vogel, 2005, Eliassen et al., 2006). However, to the best of our knowledge this is the first time that ACE inhibitory effect is described for a LF hydrolysate. In a previous work we demonstrated the *in vitro* and *ex vivo* ACE inhibiting effects of LfcinB<sub>17-31</sub> (Centeno et al., 2006). This is a synthesized 15-residue peptide derived from LfcinB, which in turn is a 25-residue antibacterial peptide isolated after pepsin cleavage of bovine LF (Bellamy et al., 1992). Interestingly the 15-residue peptide analog for LfcinB has comparable antibacterial activity to the complete LfcinB, but it is still unknown if they show similar ACE inhibitory effects. Moreover, a 6-residue peptide (LfcinB<sub>20-25</sub>) which sequence is included in that of LfcinB<sub>17-31</sub> also exhibits *in vitro* ACE inhibitory effect (Centeno et al., 2006).

#### **4. CONCLUSIONS**

The data reported here from in vitro and ex vivo experiments demonstrate that a pepsin hydrolysate of bovine LF inhibits both ACE activity and ACE-dependent vasoconstriction. Thus we have found a potential application for LF-derived peptides in the control of hypertension. However, counter-effect of LFH on angiotensin II-induced vasoconstriction deserves further research. Whether LfcinB or novel peptides are responsible for the observed ACE inhibiting effects requires further characterization studies. Nevertheless, if the use of LF hydrolysates as food additives is to be developed in the context of health-promoting functional foods, it is more likely that a raw, less purified hydrolysate would be more acceptable to the industry.



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**LIST OF ABBREVIATIONS USED**

ACE, Angiotensin I-converting enzyme; HHL, hippuryl-L-histidyl-L-leucine; LF, lactoferrin; Lfcin, lactoferricin; LfcinB, lactoferricin B; LfcinH, lactoferricin H; LFH, lactoferrin pepsin hydrolysate; SEM, standard error of the mean.

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Artículo III

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**Antihypertensive effect of a bovine lactoferrin  
hydrolysate: Identification of novel active peptides**



**ABSTRACT**

The potential of bovine lactoferrin (LF) as a source of antihypertensive peptides has been examined. For this purpose, LF pepsin hydrolysate with molecular mass lower than 3 KDa (LFH < 3KDa) was prepared and orally administered to spontaneously hypertensive rats (SHR), resulting in reduced systolic blood pressure in a significant and maintained manner up to 24 h after the administration. LFH < 3 KDa was further fractionated by semi-preparative high performance liquid chromatography and 38 peptides contained in the active fractions were identified by using an ion trap mass spectrometer. Based on peptide abundance, a total of 11 peptides were chemically synthesized and their ACE inhibitory activity tested. Only three of them, corresponding to peptides of sequences LIWKL, RPYL and LNNSRAP exerted *in vitro* inhibitory effects on angiotensin I converting enzyme (ACE) activity and had a 50% inhibitory concentration (IC<sub>50</sub>) of 0.47 μM, 56.5 μM and 105.3 μM, respectively. The three peptides also showed antihypertensive effects in SHR and remarkably the effect of LIWKL remained significant for up to 24 h post-administration, similarly to LFH < 3 KDa and the captopril control. The two most potent *in vitro* inhibitory peptides showed *ex vivo* inhibitory effect on ACE-dependent vasoconstriction as well. In conclusion, three novel LF-derived peptides and a pepsin LFH < 3KDa lower blood pressure and exhibit potential as orally effective antihypertensive compounds.

**Keywords:** Lactoferrin, lactoferrin-derived peptides, hydrolysate, ACE inhibition, ACE-dependent vasoconstriction, antihypertensive effect, bioavailability.

## **1. INTRODUCTION**

Over the past two decades increasing scientific and industrial interest has been focused on the biological properties of milk proteins, which possess additional physiological effects due to the numerous bioactive peptides that are encrypted within intact proteins. Those peptides, once released, exhibit different activities affecting the digestive, cardiovascular, immune and nervous systems (Korhonen & Pihlanto, 2006). Among them, those with blood pressure-lowering effects are receiving increasing attention due to the prevalence and importance of hypertension in the Western population (Ricci, Artacho & Olalla, 2010). The antihypertensive effect of milk protein derived peptides seems to be mainly due 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 bradykinin into an inactive peptide (Campbell, 2003) leading to blood pressure upregulation.

In this context, technological processes for the isolation and enrichment of bioactive peptides have been developed and proven effective. Basically, processing of milk proteins with (i) food grade proteolytic preparations of plant, fungal or microbial origin or digestive enzymes or (ii) fermentation of milk with proteolytic starters have been employed to release ACE inhibitory peptides. Both approaches have conducted to the development of commercial products based on casein or whey proteins with antihypertensive effects in humans (De Leo, Panarese, Gallerani & Ceci, 2009). In fact, the most popular functional foods contain the casein-derived ACE-inhibitory tripeptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), obtained by means of either milk fermentation (Nakamura, Yamamoto, Sakai & Takano, 1995) or enzymatic hydrolysis using microbial proteases (Mizuno, Nishimura, Matsuura, Gotou & Yamamoto, 2004).



In contrast to other milk proteins, there is scarce information about the potential of bovine lactoferrin (LF), a well-characterized component of milk whey, as a source of antihypertensive peptides. Recently, we have shown the vasoactive effects through ACE inhibition of a set of peptides derived from lactoferricin B (LfcinB), the well-known antimicrobial LF-derived peptide (Gifford, Hunter & Vogel, 2005). Also we described their efficacy as orally effective antihypertensive peptides (Centeno et al., 2006, Ruiz-Giménez et al., 2010). Moreover we have reported the inhibitory effects of a LF pepsin hydrolysate on ACE activity and ACE-dependent vasoconstriction using *in vitro* and *ex vivo* functional assays, respectively (Ruiz-Giménez et al., 2007). However, the antihypertensive effect of LF-derived peptides is not fully understood and according to previous studies, some other antihypertensive peptides are expected to be still identified and isolated from LF hydrolysate (Vermeirssen, van der Bent, van Camp, van Amerongen & Verstraete, 2004; Lee, Cheng, Enomoto & Nakamura, 2006).

The objective of the present study was to investigate the potential of LF as a source of antihypertensive peptides other than LfcinB-derived peptides. For this purpose, a LF pepsin hydrolysate (LFH) was ultrafiltered and fractionated. The ACE-inhibitory effect of the whole LFH (<3 KDa) permeate and each collected fraction was measured. The main peptides from the active fractions were identified by HPLC coupled to tandem mass spectrometry and chemically synthesized. Selected peptides were evaluated for their inhibitory effects on ACE activity and ACE-dependent vasoconstriction. The antihypertensive effects of individual ACE-inhibiting peptides, LFH and LFH (<3 KDa) permeate were assessed in spontaneously hypertensive rats (SHRs). Finally, the stability of ACE-inhibiting antihypertensive peptides against gastrointestinal digestion and intestinal peptidases was assessed. Both the hydrolysate and individual peptides could be applied as nutraceuticals in the context of health-promoting functional foods for the treatment of hypertension.

## **2. MATERIALS AND METHODS**

### **2.1. Lactoferrin hydrolysis and fractionation of the hydrolysate**

Bovine LF (USB Corp., Cleveland, Ohio, USA) was dissolved in distilled water at 5 % (w/vol; pH 2.5) and hydrolysed using porcine pepsin (3 % w/w, 2540 units/mg solid; Sigma Chemical Co., St. Louis, MO, USA). The hydrolysis reaction was performed at 37°C for 4 h as described previously (Ruiz-Giménez et al., 2007).

LFH was subjected to ultrafiltration through a VivaFlow 50 with a 3 KDa cut-off polyethersulfone membrane (Vivascience, Sartorius Stedim Biotech, Aubagne, France) and the permeate fractionated by reverse phase high performance liquid chromatography (RP-HPLC) on a Waters system (Waters Corporation, Milford, MA) equipped with a 1525 Binary HPLC pump, a 2996 Photodiode Array Detector, a 717 plus Autosampler in combination with a Fraction Collector III. The 3 KDa permeate was applied to a Prep Nova-Pak® HR C18, 60Å, 6 µm, 7.8 × 300 mm column (Waters). The column was developed at a flow rate of 4 mL/min. Elution was performed with a linear gradient of solvent B (acetonitrile with 0.05 % TFA) in solvent A (water with 0.05 % TFA) from 0 to 30 % B in 70 min. Samples of the whole permeate and the fractions (20 mL) were freeze-dried and kept at -20 °C until reconstitution with distilled water for determination of the protein content and *in vitro* ACE inhibitory effect as explained later on.

## **2.2. *In vitro* assay of ACE inhibitory effect**

*In vitro* ACE inhibitory activity of LFH < 3KDa, fractions and synthetic peptides was measured using the fluorescent method described by Sentandreu & Toldrá (2006) based on the hydrolysis of the internally quenched fluorescent substrate *o*-aminobenzoylglycyl-*p*-nitrophenylalanylproline (Bachem Feinchemikalien, Bubendorf, Switzerland) by the action of ACE (porcine kidney, Sigma). Protein content of peptide fractions was estimated by the bicinchoninic acid method (BCA; Sigma) using serum albumin as standard.

The IC<sub>50</sub> value was defined as the peptide concentration required to inhibit 50% of the ACE activity, and the value for each experiment was estimated by non-linear regression of the experimental data to a four-parameter logistic curve using the software package SigmaPlot v 8.02 (SPSS Inc., Chicago, IL).

## **2.3. Peptide sequencing by RP-HPLC-MS/MS**

RP-HPLC-MS/MS analysis of the ACE-inhibiting fractions was performed on an Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany) with a mediterranea<sup>TM</sup>sea<sub>18</sub> column (150 × 2.1 mm, 5µm of particle size; Teknokroma, Barcelona, Spain). The flow rate was 0.2 mL/min and the injection volume 50 µL. Peptides were eluted with a linear gradient of solvent B (acetonitrile with 0.027 % TFA) in A (acetonitrile with 0.037 % TFA) going from 0 to 20 % B over 15 min followed by a 40 min gradient from 20 to 45%. The HPLC system was connected on-line to an Esquire 3000 quadrupole ion trap (Bruker Daltonik GMBH, Bremen, Germany) equipped with an electrospray ionization source, as previously described (Contreras, Carrón, Montero, Ramos & Recio, 2009). Data Analysis (version 4.0; Bruker Daltoniks) was used to process and transform spectra to representing mass values. BioTools (version 3.1; Bruker Daltoniks) BioTools was used to process the MS<sub>n</sub> spectra, to perform peptide sequencing and to calculate theoretical masses.

The main peptides identified in the ACE-inhibiting fractions were ordered at >90% purity from GenScript Corporation (Piscataway, NJ) wherein they were synthesized by solid phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry. Synthetic peptide concentration was based on the dry weight of the peptides. These peptides were subjected to a four-step screening study to assess (1) *in vitro* inhibitory effect on ACE activity, (2) *ex vivo* inhibitory effect on ACE-dependent vasoconstriction, (3) *in vivo* antihypertensive effect, and (4) *in vitro* resistance to simulated gastrointestinal digestion and intestinal stability.

Fragments derived from LIWKL were identified by RP-HPLC-MS/MS (ProteoRed, Proteomics Facility, Centro de Investigación Príncipe Felipe, Valencia, Spain).

#### **2.4. Evaluation of potential antihypertensive peptides**

##### **2.4.1. 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*. Experimental procedures were performed 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.

##### **2.4.2. Ex vivo functional assay of ACE inhibitory effect**

Six 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/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. 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<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, and 5.6 mM glucose) as previously described (Centeno et al., 2006).

The contractile capacity of every arterial segment was checked by exposure to 50 mM KCl Ringer-Locke solution (NaCl 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: (i) control, a second challenge to angiotensin I to check for response reproducibility or (ii) treated, preincubation (20 min) with one of the LF-derived peptides (20 μM) and a second challenge to angiotensin I to check for their effect on angiotensin I-induced contraction (see Figure 2).

#### *2.4.3. In vivo assay of antihypertensive effect*

Twenty male SHRs weighing 300-350 g were used (Charles River Laboratories, Barcelona, Spain). Indirect measurement of systolic blood pressure (SBP) in awake restrained rats was carried out by the non-invasive tail-cuff method using computer-assisted NIPREM 645 equipment (Cibertec, Madrid, Spain) as previously described (Ruiz-Giménez et al., 2010). LFH (200 mg/kg), LFH < 3KDa (200 mg/kg) and individual LF-derived peptides (10 mg/kg) were orally administered by gastric intubation in 1 mL of physiological saline. The SBP was measured before peptide intake (zero time) and 1, 2, 3, 4 and 24 hours 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.

***2.5. In vitro simulated gastrointestinal digestion and intestinal stability experiments***

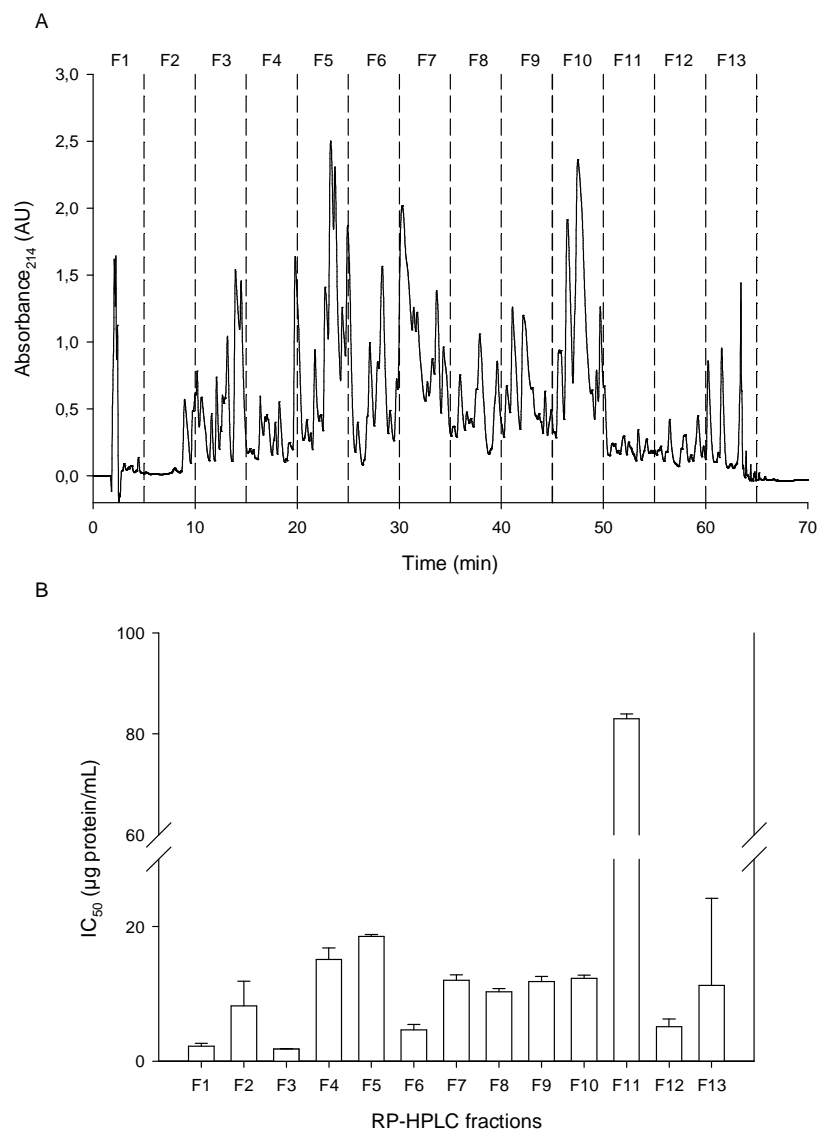
Peptides (1 mM) were subjected to a two-stage simulated gastrointestinal digestion process followed by a brush border peptidase phase as previously described (Ruiz-Giménez et al., 2010). Digests were stored at -20°C until further analysis of their ACE inhibitory activity and identification of ACE inhibiting fragments.

### **3. RESULTS**

#### **3.1. ACE inhibitory activity of lactoferrin hydrolysate and identification of major peptides**

LFH was subjected to ultrafiltration through a 3 KDa cut-off membrane and the ACE inhibitory activity of the resulting permeate (LFH < 3 KDa) was measured, showing an IC<sub>50</sub> value of 14.3 ± 3.3 µg/mL.

LFH < 3 KDa was subjected to semi-preparative RP-HPLC and the total chromatogram was divided into 13 fractions (Figure 1A). Four fractions, F1, F3, F6 and F12, exhibited the highest ACE-inhibitory activities with IC<sub>50</sub> values lower than 5 µg/mL. With the exception of F11 the rest of chromatographic fractions showed potent ACE-inhibitory activity with IC<sub>50</sub> values lower than 20 µg/mL (Figure 1B). The four most active fractions were analysed by HPLC-MS/MS and the major peptide components were sequenced (38 peptides on total, Table 1).



**Figure 1.** Fractionation by RP-HPLC of LFH < 3 KDa (panel A) and inhibitory potency on ACE, expressed as IC<sub>50</sub> value, of the collected fractions (F1-F13) (panel B). Data are expressed as mean ± SEM for a minimum of three experiments.



**Table 1.** Identification of peptides contained in the F1, F3, F6 and F12 RP-HPLC fractions of the bovine lactoferrin pepsin hydrolysate.

Fraction <sup>a</sup>	Ion for MS/MS ( <i>m/z</i> ) <sup>b</sup>	Calculated mass	Observed mass <sup>c</sup>	Protein fragment	Identified sequence
F1	605.2	604.3	604.2	f(444-448)	EGLTW
	554.2	553.2	553.2	f(225-228)	DQYE
	532.3	531.3	531.3	f(52-56)	<b>KKADA</b> <sup>d</sup>
	533.0	532.2	532.0	f(565-568)	NRED
	661.2	660.4	660.2	f(51-56)	EKKADA
	731.3	730.4	730.3	f(97-103)	<b>VVKKGSN</b> <sup>d</sup>
F3	732.3	731.3	731.3	f(456-462)	SCHTAVD
	503.2	502.3	502.2	f(53-57)	KADAV
	802.4	801.5	801.4	f(96-103)	AVVKKGSN
	787.4	786.5	786.4	f(438-444)	VVKKANE
	734.2	733.3	733.2	f(332-337)	RETAEE
	596.2	595.3	595.2	f(612-616)	<b>LHQQA</b> <sup>d</sup>
	675.2	674.4	674.2	f(271-276)	LSKAQE
	411.0	410.2	410.0	f(400-403)	<b>YTAG</b> <sup>d</sup>
	482.1	481.2	481.1	f(321-324)	GSRY
	508.2	507.2	507.2	f(538-542)	GDVAF

Table 1.(continued)

F6	803.4	802.5	802.4	f(25-31)	RMKKLGA	
	772.4	771.5	771.4	f(328-333)	LKNLRE	
	992.4	991.6	991.4	f(338-345)	VKARYTRRV	
	1018.4	1017.5	1017.4	f(255-266)	RSVDGKEDL	
	1091.5	1090.6	1090.5	f(649-658)	AKLGGRPTY	
	709.3	708.4	708.3	f(612-617)	<b>LHQQAL</b> <sup>d</sup>	
	635.3	634.4	634.3	f(133-137)	RPYLS	
	535.3	534.3	534.3	f(148-152)	<b>AVAKF</b> <sup>d</sup>	
	758.3	757.4	757.3	f(319-324)	<b>YLGSRY</b> <sup>d</sup>	
	430.1	429.3	429.1	f(669-672)	IANL	
	548.2	547.3	547.2	f(133-136)	<b>RPYL</b> <sup>d</sup>	
	F12	771.3	770.4	770.3	f(232-238)	<b>LNNSRAP</b> <sup>d</sup>
		826.5	827.5	827.5	f(404-411)	KCGLVPVL
647.4		646.4	646.4	f(402-408)	AGKCGLV	
672.4		671.4	671.4	f(266-270)	<b>LIWKL</b> <sup>d</sup>	
997.4		996.5	996.4	f(300-308)	FKDSALGFL	
702.3		701.4	701.3	f(125-130)	WVIPMG	
706.3		705.4	705.3	f(633-638)	KSETKN	
576.2		575.2	575.2	f(176-180)	EGENQ	
554.2		553.3	553.2	f(286-289)	FQLF	
738.3		737.4	737.3	f(315-321)	<b>DSALYLG</b> <sup>d</sup>	
491.3		490.2	490.3	f(635-638)	ETKN	

<sup>a</sup> Fractions are termed as in Fig. 1.

<sup>b</sup> Charge of precursor ion: 1.

<sup>c</sup> Calculated monoisotopic mass.

<sup>d</sup> Chemically synthesized peptides are labelled in bold.

### **3.2. ACE inhibitory activity of LF-derived peptides**

A total of 11 peptides (labeled in Table 1) from those identified in fractions F1, F3, F6 and F12 were chemically synthesized and their ACE inhibitory activity was tested at 20  $\mu\text{M}$  concentration. These synthesized peptides included 10 out of the 11 most abundant peptides. Although the sequence GSRY was abundant in F3 fraction, it was not evaluated because it is included within sequence YLGSR from F6 fraction. Both peptides share the last four residues at the C-terminal end, which is known to play a predominant role in competitive binding to the active site of ACE. Most of 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 (Murray & FitzGerald, 2007). Since the presence of a proline residue specifically at the antepenultimate position appears to enhance peptide binding to ACE (Rohrbach, Williams & Rolstad, 1981), the identified peptide RPYL (fraction F6) was also included in the study despite not being abundant. Only three among the eleven peptides tested showed detectable inhibitory activity at 20  $\mu\text{M}$  under our in vitro assay conditions: RPYL, LIWKL and LNNSRAP. Further concentration-response curves of these allowed the determination of their  $\text{IC}_{50}$  values as summarized in Table 2. It has to be highlighted that LIWKL exhibited an  $\text{IC}_{50}$  value of 0.47  $\mu\text{M}$ .

**Table 2.** Inhibitory potency and effects of selected LF-derived peptides on ACE activity and ACE-dependent, angiotensin I-induced arterial contractions.

Sequence	Protein fragment	IC <sub>50</sub> (μM) <sup>a</sup>	Contraction peak (%) <sup>b</sup>
Control		n.d. <sup>c</sup>	80 ± 4 (17)
RPYL	f(133-136)	56.5 ± 1.9	69 ± 3 (11)*
LIWKL	f(266-270)	0.47 ± 0.01	62 ± 3 (8)**
LNNSRAP	f(232-238)	105.3 ± 6.4	72 ± 4 (5)

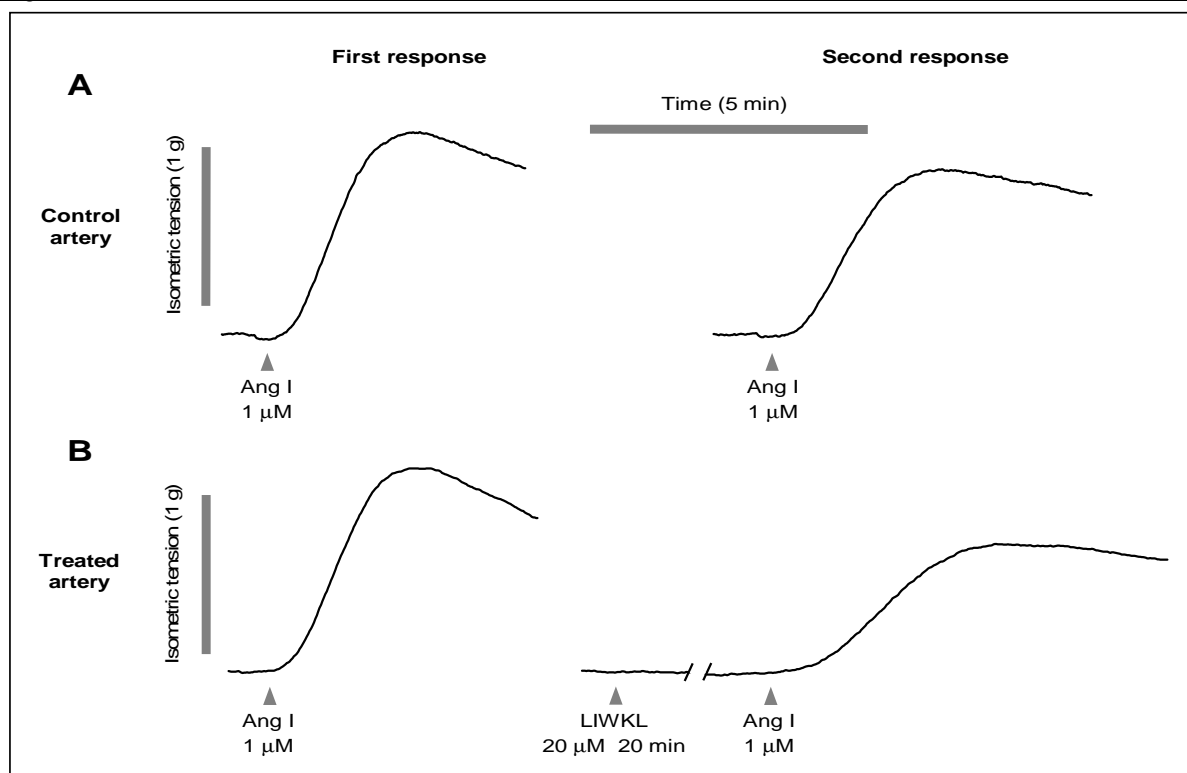
<sup>a</sup> IC<sub>50</sub> is the mean ± SEM of at least three independent experiments.

<sup>b</sup> Second contraction response to angiotensin I expressed as percentage of the first contraction in the same arterial segment. Final concentration in the assay 20 μM. Data are mean ± SEM from (n) arterial segments. \* Significantly different from control, *P*<0.05. \*\* Significantly different from control, *P*<0.01. One-way ANOVA followed by Dunnett multiple comparison tests.

<sup>c</sup> Not determined.

### **3.3. Ex vivo inhibitory effect of LF-derived peptides on ACE-dependent vasoconstriction**

The three ACE-inhibitory peptides were evaluated for ACE inhibition in vascular tissue using an *ex vivo* assay. We have previously reported that angiotensin I induces ACE-dependent vasoconstriction 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 (Centeno et al., 2006). In the present study, challenge of the arterial segments with depolarizing solution (KCl 50 mM) induced contraction averaging  $3751 \pm 160$  mg ( $n = 41$ ). Angiotensin I ( $1 \mu\text{M}$ ) induced phasic, transient contractions almost completely reproducible ( $80 \pm 4 \%$ ,  $n=17$ ) in two consecutive challenges to the same arterial segment (Figure 2A). The inhibitory effects of RPYL, LIWKL and LNNSRAP ( $20 \mu\text{M}$ ) on ACE-mediated angiotensin I-induced vasoconstriction were assessed as shown in the representative recording of Figure 2B. As summarized in Table 2, RPYL and LIWKL induced significant inhibitions when compared to the control. By contrast, although contractions to angiotensin I in arterial segments preincubated with LNNSRAP were lower than control response, the reduction was not significant. Notably, RPYL and LIWKL showed similar inhibitory effects on ACE-dependent vasoconstriction (14% and 22% response reduction over the control conditions, respectively) despite their 100-fold different *in vitro* potencies ( $\text{IC}_{50}$  values of 56.5 and  $0.47 \mu\text{M}$ , respectively).

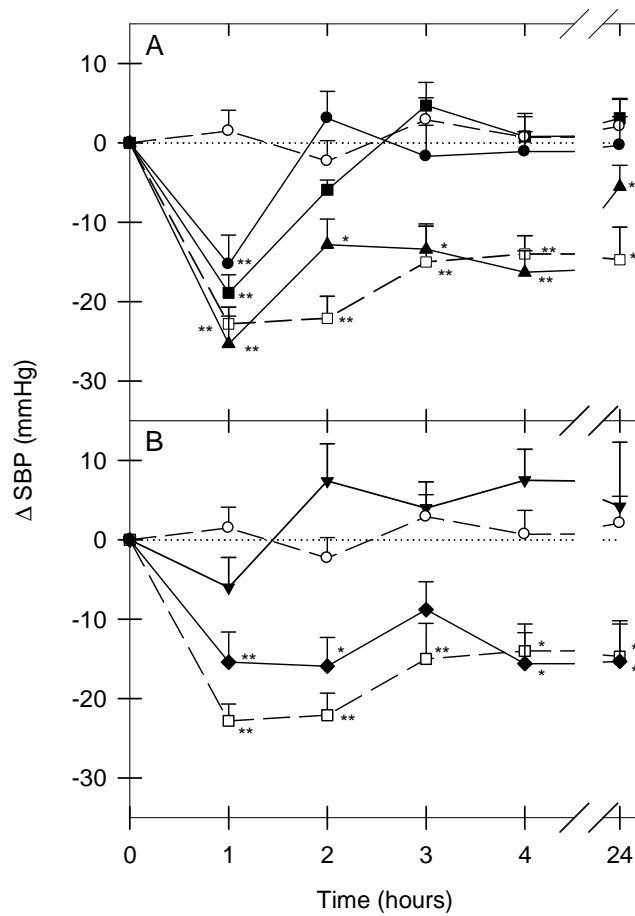


**Figure 2.** Contraction of carotid artery segments. (A) Reproducible vasoconstriction to angiotensin I. (B) Effect of LIWKL on angiotensin I-induced contraction.

**3.4. *In vivo* antihypertensive effect of LF-derived peptides in SHR**

SBP, measured by the tail-cuff method in awake SHRs, was  $202 \pm 2$  mm Hg ( $n = 45$ ). Oral administration of the three LF-derived ACE-inhibiting peptides at 10 mg/kg induced significant reductions in SBP as shown in Figure 3A, together with the lack of effect of oral saline and the antihypertensive effect of captopril (50 mg/kg) for comparison. Maximum antihypertensive effects were shown at 1 h post-administration for RPYL ( $-18.9 \pm 2.3$  mm Hg), LIWKL ( $-25.3 \pm 3.5$  mm Hg), and LNNSRAP ( $-15.3 \pm 3.7$  mm Hg). The antihypertensive effect remained significant for LIWKL up to 24 h post-administration and was comparable to that of the captopril control.

The antihypertensive effect of LFH and LFH < 3 KDa was also evaluated at a dose of 200 mg/kg. According to the time-course shown in Figure 3B, oral administration of LFH did not produce significant changes in SBP of SHRs, whereas LFH < 3 KDa significantly reduced SBP and maintained the effect up to 24 h post-administration.



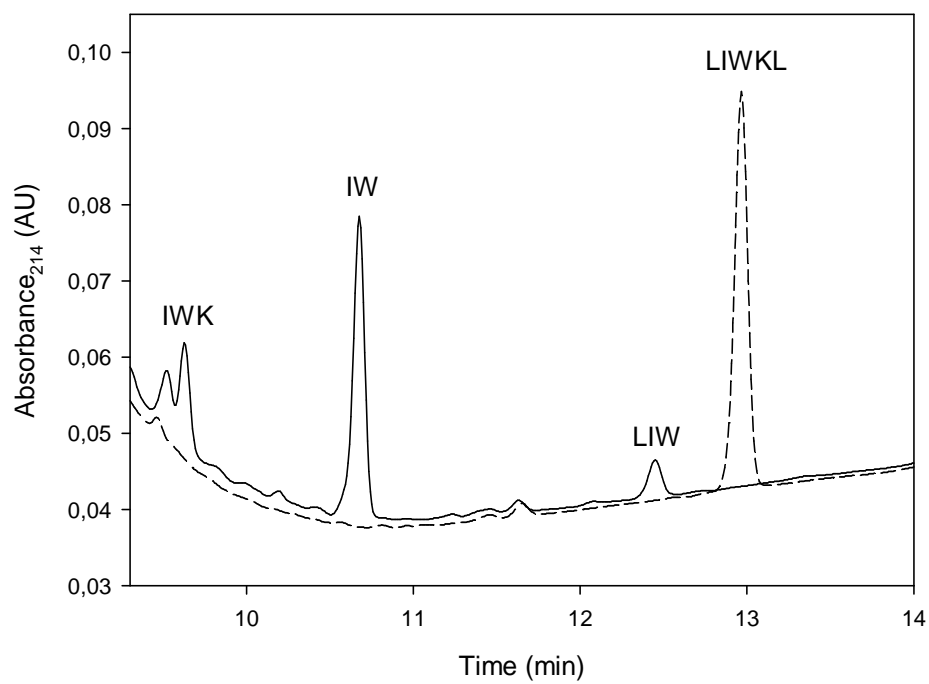
**Figure 3.** Time course of systolic blood pressure (SBP) changes after oral administration of physiological saline alone (○), captopril (□, 50 mg/kg), LFH (200 mg/kg), LFH < 3 KDa (200 mg/kg) and LF-derived peptides (10 mg/kg) to SHR. Panel A: (■) RPYL; (▲) LIWKL; (●), LNNSRAP; Panel B: (▼), LFH; (◆), LFH < 3 KDa. Pressure changes ( $\Delta$ SBP) are expressed in absolute values (mm Hg) and data are expressed as mean  $\pm$  SEM for from 5-11 determinations, \* $P$ <0.05 versus control group, \*\* $P$ <0.01 versus control group (one-way ANOVA followed by Dunnett multiple comparison tests).



### **3.5. Simulated gastrointestinal digestion and intestinal stability of LF-derived peptides**

The three selected peptides were subjected to a hydrolysis process which simulates physiological digestion due to gastric and pancreatic enzymes and brush border peptidases. The analysis of digests by RP-HPLC (data not shown) showed that RPYL was partly hydrolyzed (approximately 65% of the initial concentration of the input peptide), whereas LIWKL and LNNSRAP were almost completely hydrolyzed (hydrolysis > 90%). The resulting digests showed IC<sub>50</sub> values higher than those obtained before digestion. RPYL and LNNSRAP digests showed IC<sub>50</sub> values > 500 μM whereas the LIWKL digest showed a relatively potent ACE inhibitory activity (IC<sub>50</sub> = 7.10 ± 1.02 μM).

The potency of the LIWKL digest prompted us to identify the peptide fragments generated by the gastrointestinal digestion and to assess their ACE inhibitory potency. Figure 4 shows the peptide profile of the LIWKL digest and the three identified fragments: IWK, IW and LIW. The potency of two of them, IW (IC<sub>50</sub> = 0.41 ± 0.02 μM) and LIW (IC<sub>50</sub> = 0.22 ± 0.02 μM), was similar to that determined for the parental peptide (IC<sub>50</sub> = 0.47 ± 0.01 μM) whereas the fragment IWK showed an IC<sub>50</sub> value of 56.1 ± 1.9 μM.



**Figure 4.** RP-HPLC chromatograms of LIWKL before (dashed line) and after (solid line) being submitted to a simulated gastrointestinal digestion. Fragments IWK, IW and LIW were identified by RP-HPLC-MS/MS.

#### 4. DISCUSSION

In this study we have identified three novel LF-derived peptides with antihypertensive effects after oral administration to SHRs. These peptides were isolated from a low molecular weight fraction of a LF hydrolysate (LFH) obtained by enzymatic treatment with pepsin, which produced a significant blood pressure lowering effect ( $-15.4 \pm 3.8$  mmHg) pointing out that the antihypertensive effect may be mainly attributable to peptide components with molecular masses lower than 3 KDa as already proposed (Mullaly, Meisel & FitzGerald, 1997). Remarkably, the antihypertensive effect of LFH < 3 KDa remained significant up to 24 h post-administration. *In vivo* blood pressure-lowering effects have been also described for bovine casein and egg-white pepsin hydrolysates of molecular mass lower than 3 KDa although the antihypertensive effects of these products were transient and reverted 24 h after the administration (Miguel, López-Fandiño, Ramos & Aleixandre, 2005; Miguel, Contreras, Recio & Aleixandre, 2009).

ACE-inhibitory peptides RPYL, LIWKL and LNNSRAP isolated from the hydrolysate are reported as antihypertensive peptides for the first time. Previously, other LF-derived peptides were shown as having antihypertensive properties. A rational approach had led to the demonstration of LfcinB<sub>20-25</sub> (RRWQWR) and its derived fragment WQ as orally active antihypertensive peptides (Ruiz-Giménez et al., 2010). The potential of the inhibitory peptide LRPVAA (f(74-79)), also isolated from a pepsin hydrolysate of LF, has been suggested, although its antihypertensive effect was shown only after intravenous injection in SHR (Lee et al., 2006). None of these previous peptide sequences were identified on the present study. The sequence LIWKL share four residues with EDLIWK, a fragment isolated from a tryptic digest of LF (f(264-269)) and able to inhibit herpes simplex virus type 1 infection in association with ADRDQYELL (f(222-230)), which was isolated from the same digest (Siciliano, Rega, Marchetti, Seganti, Antonini & Valenti, 1999). Part of this latter sequence was also identified in the fraction F1 of LFH < 3KDa

(DQYE). The multifunctional properties of milk-derived ACE-inhibitory peptides has already been pointed out since they can exert both antihypertensive and antimicrobial (Ruiz-Giménez et al., 2010) or antioxidant effects (Contreras et al., 2009).

The ACE inhibition of the < 3 KDa fraction of LFH ( $IC_{50} = 14.3 \pm 3.3$   $\mu\text{g/mL}$ ) was comparable to the previously reported value of a casein permeate (5.68  $\mu\text{g/mL}$ ; Contreras et al., 2009), and as expected its *in vitro* potency was 60 times higher than the unfractionated LFH previously characterized (950  $\mu\text{g/mL}$ ; Ruiz-Giménez et al., 2007).

The ACE inhibitory potency of the three peptides isolated from LFH varied over a 200-fold range (Table 2). The two peptides RPYL and LNNSRAP had  $IC_{50}$  values higher than those reported for LfcinB inhibitory peptides ( $IC_{50}$  values from 2.3 to 26.7  $\mu\text{M}$ ; Ruiz-Giménez et al., 2010) and LRPVAA ( $IC_{50} = 4.14$   $\mu\text{M}$ ; Lee et al., 2006). However, the three peptides identified in this study had more similar behaviors on *ex vivo* (Table 2) and oral administration (Figure 2) assays, pointing out to the need of thorough full characterization of potentially antihypertensive peptides. The lack of correlation observed between the inhibitory effects on ACE dependent vasoconstriction and the inhibitory effects observed *in vitro* on ACE activity have been also reported for lactoferricin B-derived peptides (Ruiz-Giménez et al., 2010) and casein hydrolysates (Rousseau-Ralliard et al., 2010). Although this discrepancy deserves further research, a methodological detail could at least in part account for it. The *in vitro* test was carried out with porcine ACE, while rabbit arteries were used in the *ex vivo* test, and variations in the inhibition profiles of ACE from different species have been reported (Vazeux, Cotton, Cuniasse & Dive, 2001).

LIWKL is the most potent LF-derived ACE inhibitory peptide described so far ( $IC_{50} = 0.47 \pm 0.01$   $\mu\text{M}$ ). Nevertheless its  $IC_{50}$  value is still far from the  $IC_{50}$  (0.022  $\mu\text{M}$ ) of the synthetic ACE inhibitor captopril (Ondetti, Rubin & Cushman, 1977). LIWKL also exerted the highest antihypertensive effect on SHRs in our study ( $-25.3 \pm 3.5$  mm Hg), which is

12.1% reduction from baseline SBP. As occurred with LFH < 3 KDa and the captopril control, the antihypertensive effect of LIWKL remained significant for up to 24 h post-administration. The peptide also showed inhibitory effects on ACE-dependent, angiotensin I-induced contractions in the carotid artery (Table 2) adding functional evidence for a mechanism of antihypertensive effect based in ACE inhibition. LIWKL was completely hydrolysed in the simulated digestion. Further data strongly suggest that the *in vivo* antihypertensive effect may be partly due to derivative fragments, since the digest showed a potent inhibitory activity ( $IC_{50} = 7.10 \pm 1.02 \mu M$ ), and two of the fragments identified (IW and LIW) showed an *in vitro* inhibitory activity as potent as that of the intact peptide. The sequence IW was among the most potent dipeptide ACE inhibitors described in the pioneering studies of Cheung, Wang, Ondetti, Sabo & Cushman (1980). To what extent LIWKL or its derived fragments are responsible of the *in vivo* effects deserves further research.

Although RPYL and LNNSRAP exerted antihypertensive efficacy, the effect was lost at 2 h post-administration. This result might be related to gastrointestinal digestion and the release of non active peptides as showed by the  $IC_{50}$  values of the corresponding digests. In contrast to the inhibitory effect on ACE-dependent vasoconstriction showed by RPYL, LNNSRAP did not show such effect, suggesting a mechanism for the antihypertensive effect other than inhibition of ACE-related vasoactive effects. This result is similar to our previous data with the fragment WQ derived from the gastrointestinal digestion of LfcinB<sub>20-25</sub> (Ruiz-Giménez et al., 2010) and also with  $\alpha_{S1}$ - and  $\alpha_{S2}$ -casein hydrolysates which failed to inhibit angiotensin I-induced vasoconstriction in aortic tissue (Rousseau-Ralliard et al., 2010). Increasing evidence is being provided that mechanisms other than ACE inhibition may be also involved in the blood pressure-lowering effect exerted by many food derived peptides (López-Fandiño, Otte & van Camp, 2006). It has been described that a casein hydrolysate containing VPP and IPP improves vascular endothelial function independent of blood pressure lowering effects (Hirota et al., 2007). Previously, it was reported that the

modulation of endothelin-1 (ET-1), a peptide that has powerful vasoconstrictor and pressor properties (Kedzierski & Yanagisawa, 2001), may play a role in the antihypertensive effects of the ACE-inhibitory milk-derived peptide lactokinin (Maes et al., 2004). Recently, we have shown that some ACE-inhibitory LfcinB-derived peptides can also act as inhibitors of the endothelin converting enzyme, the key peptidase in the production of ET-1 (Fernández-Musoles et al., 2010). In the same way, different cardiovascular benefits have been recently described after the oral administration of an antihypertensive casein hydrolysate to SHR. It improved aorta and mesenteric acetylcholine relaxations, increased the endothelial nitric oxide synthase expression in aorta and decreased left ventricular hypertrophy, accompanied by a significant decrease in interstitial fibrosis (Sánchez et al., 2011).

## **5. CONCLUSIONS**

We have shown that hydrolysis of LF with pepsin generates antihypertensive peptides identified as sequences RPYL, LIWKL and LNNSRAP. Also, data reported here demonstrate that LFH of molecular mass lower than 3 KDa exerts antihypertensive effects suggesting its potential application as nutraceutical in the treatment of hypertension. In this context, it is more likely that the hydrolysate would be better accepted by the industry and consumers than the use of pure peptides, which would be more conceivable for pharmaceutical applications. Whether only the three sequences identified are responsible for the observed ACE inhibiting and antihypertensive effects of LFH < 3 KDa requires further characterization studies. Mechanisms other than ACE inhibition potentially involved in the antihypertensive effect of LF-derived peptides deserve further research.

**ACKNOWLEDGEMENTS**

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Artículo IV

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**Novel antihypertensive hexa- and heptapeptides with  
ACE-inhibiting properties: From the in vitro ACE assay  
to the spontaneously hypertensive rat**



**ABSTRACT**

Bioactive ACE inhibiting peptides are gaining interest in hypertension treatment. We have designed and screened six synthetic heptapeptides (PACEI48 to PACEI53) based on two hexapeptide leads (PACEI32 and PACEI34) to improve ACE inhibitory properties and assess their antihypertensive effects. ACE activity was assayed in vitro and ex vivo. Selected peptides were administered to spontaneously hypertensive rats (SHRs) and normotensive Wistar-Kyoto (WKY) rats. In vitro cytotoxicity was assessed with the MTT reduction test. The six heptapeptides at low micromolar concentration produced different degrees of in vitro inhibition of ACE activity using the synthetic substrate HHL or the natural substrate angiotensin I; and ex vivo inhibition of ACE-dependent, angiotensin I-induced vasoconstriction, but not angiotensin II-induced vasoconstriction. Oral administration of the hexapeptide PACEI32L, and the heptapeptides PACEI50L and PACEI52L, induced reductions in systolic blood pressure lasting up to 3 h in SHRs but not in WKY rats. Intravenous injection of PACEI32L and PACEI50L, but not PACEI52L, induced acute transient reductions in mean blood pressure of SHRs. D-amino acid peptides showed five-fold less ACE inhibitory potency, no inhibitory effect on angiotensin I-induced vasoconstriction, and antihypertensive effect in SHRs after i.v. injection, but not after oral administration. The toxicity of peptides to reduce the viability of cultured cells was in the millimolar range. In conclusion, we have obtained novel rationally designed heptapeptides with improved ACE inhibitory properties when compared to lead hexapeptides. One selected hexapeptide and two heptapeptides show oral antihypertensive effects in SHRs and appear safe in cytotoxicity assays.

**Keywords:** angiotensin converting enzyme; ACE inhibitor; vasoconstriction; hypertension; spontaneously hypertensive rat; antihypertensive peptide.

## **1. INTRODUCTION**

The increase in cardiovascular risk inherent to hypertension leads to premature morbidity and mortality [10]. Prolonged blood pressure lowering through lifestyle interventions or pharmacological treatments is associated with reductions in the risks of stroke (30–40%) and coronary events (20%), and appears to reduce to a lesser degree the incidences of other complications [31]. Since dysfunctions in the renin-angiotensin-aldosterone system (RAAS) are involved in the pathophysiology of hypertension [3], several antihypertensive drugs target on this system at different points of the cascade: renin inhibitors, angiotensin converting enzyme (ACE) inhibitors, AT1 receptor blockers and aldosterone receptor antagonists [31]. ACE inhibition is also the goal of antihypertensive peptides derived from plant and animal proteins such as milk, soy or fish. These peptides can be enzymatically released from precursor proteins during food processing and gastrointestinal digestion. They may be generated in or incorporated into functional foods for the development of 'natural' beneficial health products, some of which are currently on the market or in development [7]. Lactotripeptides (IPP and VPP), the best characterized ACE inhibitor peptides found in fermented milk, have shown blood pressure-lowering effects in most clinical trials [2], but negative results have also been reported [8,26].

An alternative approach is the development of de novo or rationally designed synthetic ACE inhibitor peptides [15,11]. Synthetic peptides present some advantages over naturally produced ones regarding the characterization of antihypertensive properties, such as the use of sequence related peptides aimed at deciphering structure-activity relationships, improving bioactivity or bioavailability, and elucidating the mechanism of action. In addition, the synthetic approach could lead to the identification of novel candidate peptides.



In a previous study [4], we screened a series of sequence-related hexapeptides with limited homology to the antimicrobial motif of bovine lactoferrin, and sharing all of them a C-terminal FW dipeptide motif commonly found in many ACE inhibitor peptides [5,32]. The inhibitory effect of these peptides on ACE was studied by using both in vitro and ex vivo assays and we identified two hexapeptides (PACEI32 and PACEI34) with potential antihypertensive properties.

The objective of the present work was the design and screening of heptapeptides based on the PACEI32 and PACEI34 hexapeptide leads in order to improve ACE inhibitory effect. In addition, we carried out experiments with selected peptides in spontaneously hypertensive rats (SHRs) to confirm the antihypertensive potential and in cell culture to discard undesirable cytotoxic effects.

## 2. MATERIALS AND METHODS

### 2.1. Peptides and other compounds

Peptides (Table 1) were purchased at >90% purity from GenScript Corp. (Piscataway, NJ). They were synthesized and handled as previously described [4].

**Table 1.** Amino acid sequences of peptides.

	Peptide	Sequence
L-stereoisomers	PACEI32L	Ac-RK <u>WH</u> FW-NH <sub>2</sub>
	PACEI34L	Ac-RK <u>WL</u> FW-NH <sub>2</sub>
	PACEI48L	Ac-RK <u>WFHL</u> W-NH <sub>2</sub>
	PACEI49L	Ac-RK <u>WFLH</u> W-NH <sub>2</sub>
	PACEI50L	Ac-RK <u>WHFL</u> W-NH <sub>2</sub>
	PACEI51L	Ac-RK <u>WHL</u> FW-NH <sub>2</sub>
	PACEI52L	Ac-RK <u>WLFH</u> W-NH <sub>2</sub>
	PACEI53L	Ac-RK <u>WLHF</u> W-NH <sub>2</sub>
	Angiotensin I	DRVYI <u>HPFHL</u>
D-stereoisomers	PACEI32D	Ac-rk <u>wh</u> fw-NH <sub>2</sub>
	PACEI50D	Ac-rk <u>whfl</u> w-NH <sub>2</sub>

The hexa- and heptapeptides are named PACEI (from peptide and ACE inhibitor). The L-amino acids are shown in uppercase letters, and the D-amino acids in lowercase letters. Residues equal to those three C-terminal of angiotensin I are underlined.

Hippuryl-L-histidyl-L-leucine (HHL), angiotensin converting enzyme (ACE, EC 3.4.15.1) from porcine kidney, captopril and bradykinin fragment 1-5 were purchased from Sigma (Madrid, Spain). Angiotensin I, angiotensin II and bradykinin were provided by Calbiochem Co. (La Jolla, CA).

## **2.2. *In vitro* assay of inhibitory effect on ACE**

Peptides were assayed *in vitro* for their capacity to inhibit the ACE activity using HHL, angiotensin I, or bradykinin as substrates and quantifying the reaction products hippuric acid, angiotensin II, or bradykinin fragment 1-5, respectively, by RP-HPLC following the protocol described previously [4].

## **2.3. *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*.

Experimental procedures were conducted in accordance with the Spanish legislation on 'Protection of Animals used for Experimental and other Scientific Purposes' and the study was approved by the 'Ethics Committee for Animal Welfare' of the Hospital La Fe.

## **2.4. *Ex vivo* functional assay of inhibitory effect on ACE**

Twelve male New Zealand White rabbits (Technology Transferring Center, Polytechnic University of Valencia, Spain), weighing 2.5-3 kg, were killed by injection of 25 mg/kg sodium thiopental (Tiobarbital Braun<sup>®</sup>, 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.

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<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, and 5.6 mM glucose) as previously described [23].

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 with one of the peptides (20  $\mu$ M) and a second challenge to angiotensin I to check for their effect on angiotensin I-induced contraction. Additionally, some arterial segments were subjected to the first or second protocol but with angiotensin II (1  $\mu$ M) instead of angiotensin I. This was a suitable protocol for checking and discriminating peptide effects on either ACE activity or angiotensin receptors mediating vasoconstriction to angiotensins [4]. Finally, some arterial segments were subjected to two successive challenges to 50 mM KCl with or without one of the peptides (20  $\mu$ M) in between to check for nonspecific effects of the peptides on arterial contractile capacity.

### **2.5. *In vivo* assay of antihypertensive effect**

Thirty two male SHR<sub>s</sub> weighing 300-350 g were used, together with four male Wistar Kyoto (WKY) rats as the normotensive control (Charles River Laboratories Spain, S.A).

Indirect measurement of systolic blood pressure (SBP) in awake restrained rats was carried out by the non-invasive tail-cuff method using computer-assisted NIPREM 645 equipment (Cibertec, Madrid, Spain) as previously described [24]. The peptides were orally administered by gastric intubation at a dose of 10 mg/kg in 1mL of physiological saline. The SBP was measured before peptide intake (zero time) and 1, 2, 3, 4 and 24 hours after intake. Physiological saline alone (1mL) and captopril (50 mg/kg) served as negative and positive controls respectively. Each determination of SBP was obtained by averaging three consecutive and successful measurements without disturbance of the signal.

Direct measurement of arterial blood pressure (ABP) in anesthetized rats was carried out by the femoral artery cannulation method [25]. Anesthesia was induced by intraperitoneal injection of 5 mg/kg diazepam (Almirall Prodesfarma, Barcelona, Spain) and 100 mg/kg ketamine (Ketolar, Parke-Davis, El Prat de Llobregat, Barcelona, Spain)

and maintained with 0.5-1% sevoflurane (Sevorane, Abbott Laboratories, Madrid, Spain) in a mixture of 70% N<sub>2</sub>O and 30% O<sub>2</sub>. The left femoral vein was also cannulated for peptide injection. The peptides were administered at doses of 0.5, 0.7 or 1 mg/kg in 1 mL of physiological saline. The mean ABP of a ten minute interval was measured before peptide injection (baseline value) and at maximum effect after peptide injection.

### **2.6. MTT assay of cytotoxic effect**

Primary cultured rat hepatocytes and two cells lines (3T3 and HepG2) were seeded on 96-well microtitre plates to obtain an assay density of  $25 \times 10^3$  viable cells/well, and treatment with increasing concentrations (100  $\mu$ M – 10 mM) of the peptides started at 24 h of culture. After 24 h of exposure cytotoxic effects were measured by using the MTT [(3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction test [21].

### **2.7. Statistics**

Data are mean $\pm$ SEM. Statistical analysis was carried out as indicated in results, figure captions or table footnotes.  $P < 0.05$  was considered significant.

### 3. RESULTS

#### 3.1. *In vitro* inhibitory effects of heptapeptides on ACE activity

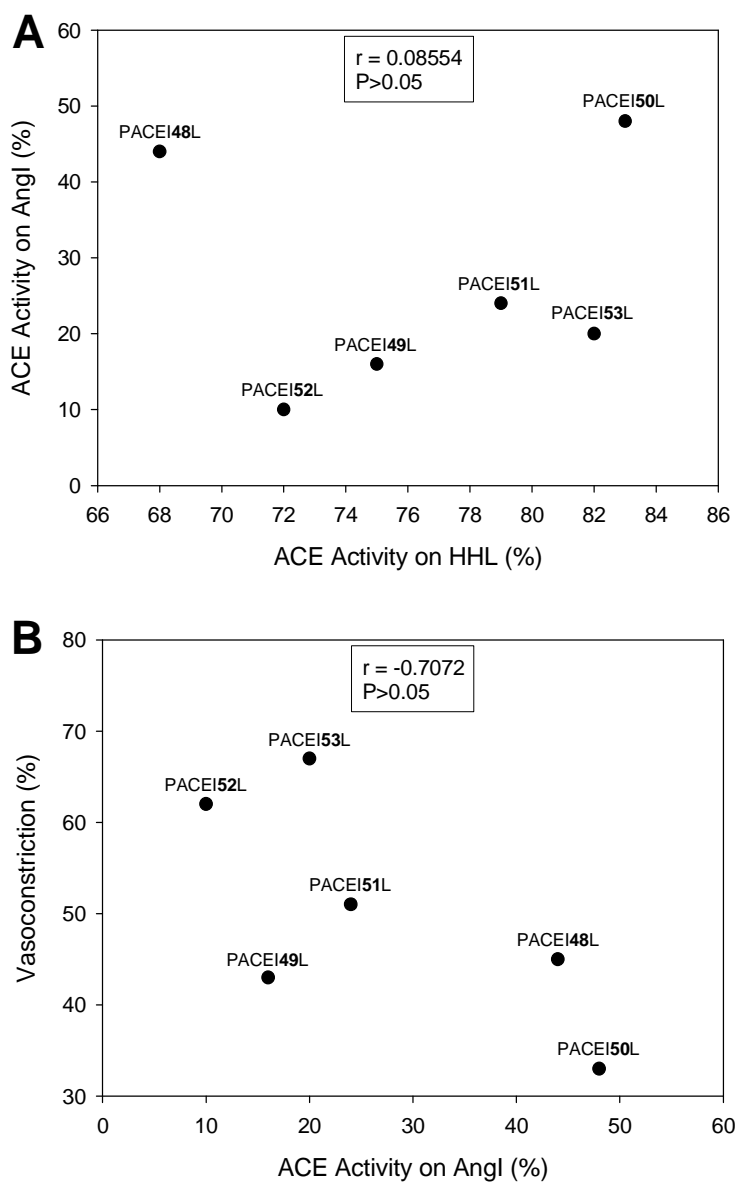
Based on hexapeptides PACEI32 and PACEI34 [4], a second peptide generation of heptapeptides was synthesized (Table 1). This new generation was obtained by combinations of residues in positions 4, 5 and 6. These combinations are based in the structural studies indicating that the C-terminal positions are the important ones for the inhibitory effect of peptides on ACE [5,32]. F, H and L residues were used because they are preceding the C-terminal W residue in bioactive PACEI32 and PACEI34, and also they are the three last C-terminal residues of angiotensin I (natural substrate of ACE).

The inhibitory effects of 20  $\mu$ M heptapeptides on ACE activity were assessed by using the synthetic substrate HHL or the natural substrate angiotensin I (Table 2). The six heptapeptides (PACEI48L, PACEI49L, PACEI50L, PACEI51L, PACEI52L, and PACEI53L) showed significant ACE inhibitory effects regardless of the substrate used. With HHL as substrate, significantly different residual ACE activities ranged from 68% for PACEI48L to 83% for PACEI50L, whereas with angiotensin I they ranged from 10% for PACEI52L to 48% for PACEI50L. When the two substrates were compared, residual ACE activities were for all heptapeptides significantly lower with angiotensin I. However, there was no good significant correlation between ACE activities on HHL and on angiotensin I for the six heptapeptides. As can be seen in Fig. 1A, PACEI48L broke the tendency to a positive correlation pointed by the other five heptapeptides. On the other hand, none of the heptapeptides at 20  $\mu$ M inhibited ACE activity when bradykinin, another ACE natural substrate, was used (data not shown).

**Table 2.** Effects of heptapeptides on ACE activity.

Peptide (20 $\mu$ M)	ACE activity (%)	
	HHL	Ang I
PACEI48L	68 $\pm$ 5 (7) (a)	44 $\pm$ 3 (5) (A)
PACEI49L	75 $\pm$ 5 (4) (a, b, c)	16 $\pm$ 1 (8) (B, C)
PACEI50L	83 $\pm$ 3 (6) (b)	48 $\pm$ 3 (6) (A)
PACEI51L	79 $\pm$ 1 (4) (b, c)	24 $\pm$ 1 (6) (B)
PACEI52L	72 $\pm$ 2 (8) (a, c)	10 $\pm$ 1 (8) (C)
PACEI53L	82 $\pm$ 3 (4) (b)	20 $\pm$ 1 (5) (B)

ACE activity was measured by using both synthetic (HHL) and natural (Ang I) substrates, and is expressed as percent ACE residual activity with respect to a control without peptide (100%). Data are mean  $\pm$  SEM from (n) experiments and were subjected to two-way ANOVA followed by Bonferroni post-tests. All data are significantly lower than their respective controls without peptide ( $P < 0.01$ ; test on unshown absolute values of ACE activity). Data with the same letter are not significantly different ( $P > 0.05$ ). Data for all peptides are significantly lower with Ang I when compared to HHL ( $P < 0.01$ ).



**Figure 1.** (A) Correlation analysis of in vitro residual ACE activities, using HHL or angiotensin I as substrate, after incubation with each of the six heptapeptides. (B) Correlation analysis of in vitro residual ACE activity, using angiotensin I as substrate, and ex vivo vasoconstrictor response to angiotensin I, after incubation with each of the six heptapeptides. Pearson correlation coefficients ( $r$ ) and two tailed  $P$  values are given in the inserts.



The inhibitory potency, indicated by  $IC_{50}$ , was determined for the six heptapeptides with angiotensin I as substrate (Table 3). Except for PACEI48L, the rest of heptapeptides showed  $IC_{50}$  values below 10  $\mu$ M. The influence of amino acid stereoisomerism on the ACE inhibitory effect of peptides was assessed by using PACEI50D, the D-amino acid enantiomeric counterpart of PACEI50L. Inhibitory potency of PACEI50D was more than five-fold reduced with respect to that of PACEI50L ( $IC_{50}$  values of  $45.7 \pm 5.2 \mu$ M and  $8.2 \pm 1.0 \mu$ M, respectively,  $n = 3$ ; significantly different,  $P < 0.01$ , Student's t test).

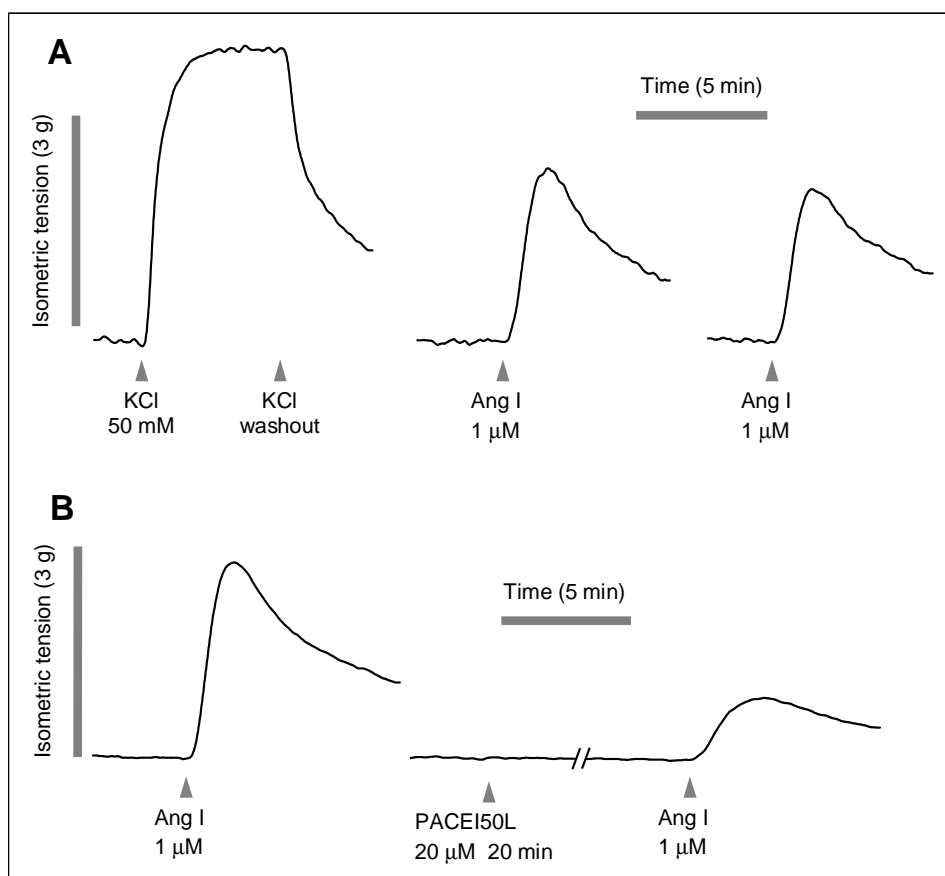
**Table 3.** Inhibitory potency of heptapeptides on ACE activity.

Peptide	$IC_{50}$ ( $\mu$ M)
PACEI48L	$16.4 \pm 3.9$ (3) (a)
PACEI49L	$5.9 \pm 0.4$ (4) (b)
PACEI50L	$8.2 \pm 1.0$ (3) (b)
PACEI51L	$6.3 \pm 0.6$ (3) (b)
PACEI52L	$3.0 \pm 0.2$ (4) (b)
PACEI53L	$5.1 \pm 0.6$ (3) (b)

Inhibitory potency is expressed as  $IC_{50}$  defined as the peptide concentration expected to inhibit 50% of the ACE activity and calculated for each experiment by nonlinear regression of the experimental data to a four-parameter logistic curve. Data are mean  $\pm$  SEM from (n) experiments and were subjected to one-way ANOVA followed by Student-Newman-Keuls post-test. Data with the same letter are not significantly different ( $P > 0.05$ ).

### 3.2. Ex vivo inhibitory effect of heptapeptides on angiotensin I-induced 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 (Fig. 2A). Average active tone amounted to  $4364 \pm 132$  mg ( $n = 106$ ). Angiotensin I ( $1 \mu\text{M}$ ) induced phasic, transient contractions that were almost completely reproducible (87%) in two consecutive challenges to the same arterial segment (Fig. 2A).



**Figure 2.** Contraction of carotid artery segments. (A) Effect of high-KCl to check for tissue viability, and reproducible vasoconstriction to angiotensin I. (B) Effect of PACEI50L on angiotensin I induced contraction.

The inhibitory effects of 20  $\mu$ M heptapeptides on angiotensin I-induced vasoconstriction were assessed as shown in the representative recording of Fig. 2B. All the six heptapeptides produced significant inhibition when compared to control vasoconstriction (Table 4). The degree of inhibition was significantly different among peptides, ranging from 23% response reduction over the control conditions for the weakest PACEI53L to 62% response reduction for the strongest PACEI50L. Fig. 1B shows the lack of correlation between the inhibitory effects of the six heptapeptides on in vitro ACE activity and on ex vivo angiotensin I-induced vasoconstriction.

**Table 4.** Effects of heptapeptides on ACE-dependent angiotensin I-induced arterial contractions.

Peptide (20 $\mu$ M)	Contraction Peak (%)
Control	87 $\pm$ 3 (33)
PACEI48L	45 $\pm$ 9 (9) (a, b)*
PACEI49L	43 $\pm$ 4 (9) (a, b)*
PACEI50L	33 $\pm$ 5 (9) (a)*
PACEI51L	51 $\pm$ 4 (9) (a, b, c)*
PACEI52L	62 $\pm$ 5 (9) (b, c)*
PACEI53L	67 $\pm$ 6 (9) (c)*

Second contraction response to angiotensin I expressed as percentage of the first contraction in the same arterial segment. Data are mean  $\pm$  SEM from (n) arterial segments and were subjected to one-way ANOVA followed by Student-Newman-Keuls post-test. Data with the same letter are not significantly different ( $P>0.05$ ).

\*Significantly different from control,  $P<0.01$ .

Given the stereoselectivity of the inhibitory effects of PACEI50 on ACE activity, PACEI50D was also assayed against angiotensin I-induced vasoconstriction. PACEI50D did not induce inhibition of the vasoconstriction ( $105 \pm 6\%$ ,  $n = 6$ ) when compared to control ( $98 \pm 7\%$ ,  $n = 4$ ;  $P > 0.05$ , Student's t-test) and in contrast to significant inhibition produced by PACEI50L (Table 4). Moreover, the effect of PACEI50L was assayed against angiotensin II-induced contraction of carotid artery. Response to angiotensin II obtained after PACEI50L incubation ( $71 \pm 2\%$ ,  $n = 6$ ) was not significantly different to that obtained in control conditions ( $84 \pm 8\%$ ,  $n = 3$ ;  $P > 0.05$ , Student's t-test). Finally, the effect of PACEI50L was assayed against KCl-induced vasoconstriction. Contrary to angiotensins, in control conditions the second vasoconstriction to KCl was higher than the first response in the same arterial segment ( $137 \pm 9\%$ ,  $n = 4$ ), and this increase was not significantly different after PACEI50L incubation ( $144 \pm 9\%$ ,  $n = 4$ ;  $P > 0.05$ , Student's t-test).

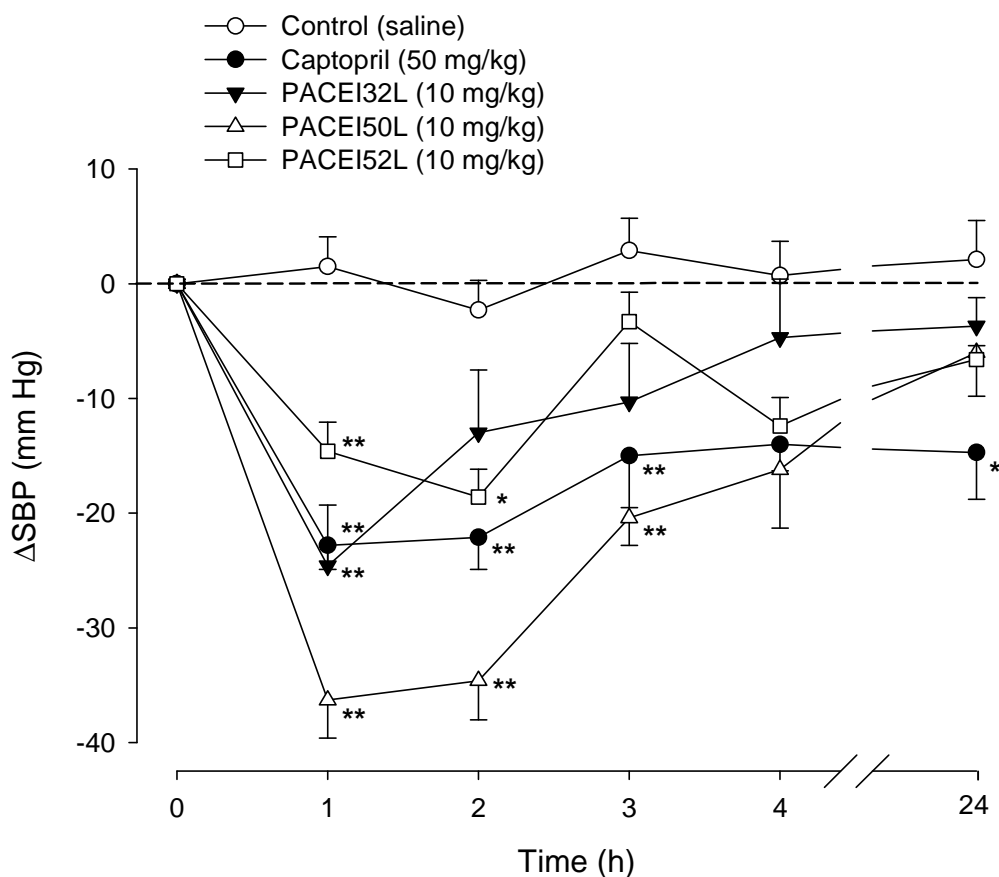
### **3.3. *In vivo* antihypertensive effect of peptides in rats**

The lack of correlation between in vitro and ex vivo results prompted the need for in vivo experiments to directly assess the potential antihypertensive effects of peptides in SHR. Three peptides were selected: (a) PACEI32L, as the reference hexapeptide previously reported with the best inhibitory effect on ACE-mediated vasoconstriction; (b) PACEI50L, as the heptapeptide with the best inhibitory effect on ACE-mediated vasoconstriction; and (c) PACEI52L, as the heptapeptide with the best inhibitory effect on in vitro ACE activity. The D-amino acid peptides were also used to check on stereoselectivity in the in vivo effects.

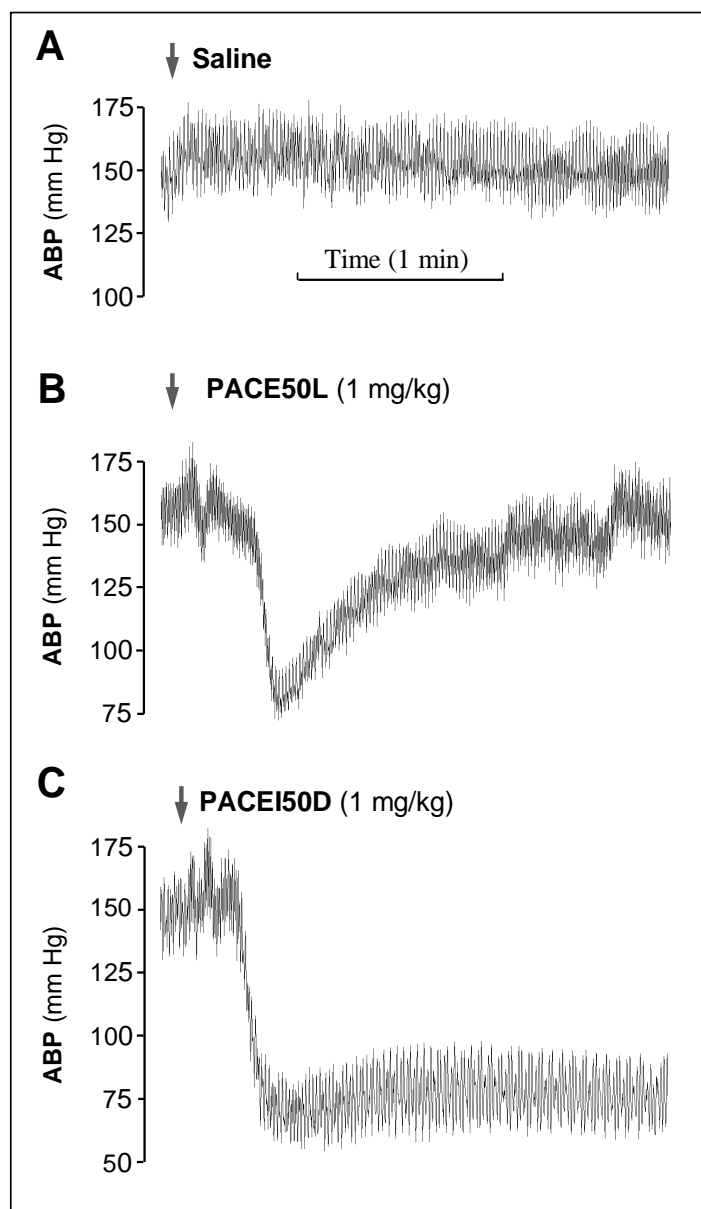
Systolic blood pressure (SBP), measured by the tail-cuff method in awake SHR, was  $201 \pm 2$  mm Hg ( $n = 59$ ). Oral administration of PACEI32L, PACEI50L or PACEI52L at 10 mg/kg induced significant reductions in SBP which time-courses are shown in Fig. 3, together with the lack of effect of oral saline and the antihypertensive effect of captopril (50 mg/kg) for comparison. Maximum antihypertensive effects were shown at

1 h post-administration for PACEI32L ( $-24.6 \pm 5.3$  mm Hg) and PACEI50L ( $-36.3 \pm 3.3$  mm Hg), whereas PACEI52L produced its maximum effect at 2 h ( $-18.6 \pm 5.3$  mm Hg). The antihypertensive effect remained significant for PACEI50L up to 3 h post-administration. Oral administration of PACEI32D or PACEI50D at 10 mg/kg did not produce changes in SBP of SHR (data not shown). WKY rats, the normotensive control strain, showed SBP of  $116 \pm 2$  mm Hg ( $n = 23$ ). In these rats, oral administration of PACEI32L, PACEI50L or PACEI52L at 10 mg/kg did not modify SBP (data not shown).

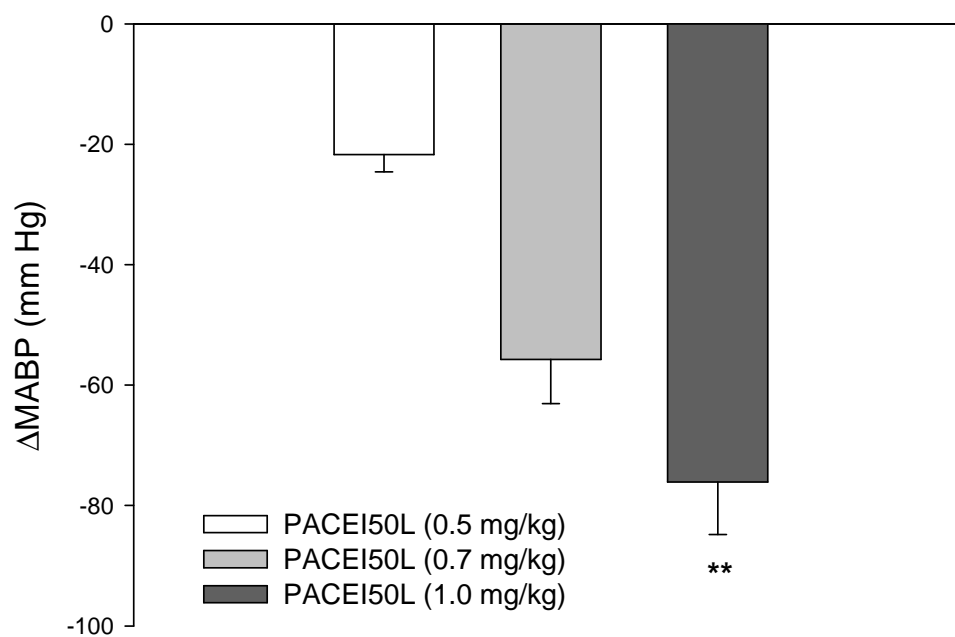
Mean arterial blood pressure (MABP), measured by femoral artery cannulation in anesthetized SHRs, was  $158 \pm 5$  mm Hg ( $n = 29$ ). Intravenous injection of PACEI32L or PACEI50L at 1 mg/kg induced acute transient reductions in MABP (a representative recording is shown in Fig. 4B), while PACEI52L at 1 mg/kg did not modify MABP. The effect of PACEI50L (0.5, 0.7 and 1 mg/kg) on MABP was dose-dependent (Fig. 5). Injection of PACEI32D or PACEI50D at 1 mg/kg induced maintained reductions in MABP lasting for at least 1 h (representative recording in Fig. 4C). The magnitude of the antihypertensive effects of PACEI32 and PACEI50 are summarized in Table 5, together with the lack of effect of saline or PACEI52L injections.



**Figure 3.** Time course of systolic blood pressure (SBP) changes after oral administration of physiological saline alone, captopril (50 mg/kg), hexapeptide PACEI32L and heptapeptides PACEI50L and PACEI52L (10 mg/kg) to SHR. Pressure changes from baseline (time 0) are expressed in absolute values (mm Hg). Data are mean  $\pm$  SEM from 5-11 determinations and were subjected to one-way ANOVA followed by Dunnett post-test; \*Significantly different from control,  $P < 0.05$ ; \*\*Significantly different from control,  $P < 0.01$ .



**Figure 4.** Arterial blood pressure recordings in the femoral artery of SHRs. Changes induced by i.v. injection of (A) physiological saline, (B) PACEI50L (1 mg/kg), and (C) PACEI50D (1 mg/kg).



**Figure 5.** Dose-dependent effects of PACEI50L (0.5, 0.7 and 1 mg/kg; i.v.) on mean arterial blood pressure (MABP) in SHR. Data are mean  $\pm$  SEM from 3-8 determinations and were subjected to one-way ANOVA followed by Student-Newman-Keuls post-test; \*\*Significantly different from 0.5 mg/kg,  $P < 0.01$ .



**Table 5.** Effects of intravenous peptides on mean arterial blood pressure in SHRs.

Peptide (1 mg/kg)	$\Delta$ MABP (mm Hg)
Control (saline)	5.9 $\pm$ 5.2 (5)
PACEI50L	-76.1 $\pm$ 8.7 (8) (a)*
PACEI50D	-83.5 $\pm$ 6.7 (3) (a)*
PACEI52L	-1.6 $\pm$ 2.1 (5) (b)
PACEI32L	-76.4 $\pm$ 10.2 (5) (a)*
PACEI32D	-97.7 $\pm$ 15.2 (3) (a)*

$\Delta$ MABP, mean arterial blood pressure change from baseline, before peptide injection, expressed in absolute values (mm Hg). Data are mean  $\pm$  SEM from (n) determinations and were subjected to one-way ANOVA followed by Student-Newman-Keuls post-test. Data with the same letter are not significantly different ( $P>0.05$ ).

\*Significantly different from control,  $P<0.01$ .

### 3.4. *In vitro* effects of peptides on cell viability

Safety of hexapeptide PACEI32L and heptapeptide PACEI50L, both with antihypertensive effects after oral or i.v. administration, was assayed in three cell types (primary rat hepatocytes, 3T3 cells and HeptG2 cells). The potencies of the two peptides at reducing cell viability are summarized in Table 6. Although there was significantly different cellular toxicity depending on both the peptide and the cell type, IC<sub>50</sub> values were all in the millimolar range, from 0.41 ± 0.07 mM for PACEI50L on primary rat hepatocytes to 5.76 ± 0.92 mM for PACEI32L on HeptG2 cells.

**Table 6.** Effects of peptides on viability of cultured cells.

Peptide	IC <sub>50</sub> (mM)		
	Primary rat hepatocytes	3T3 cells	HeptG2 cells
PACEI32L	1.23 ± 0.13*	3.35 ± 0.89*	5.76 ± 0.92*
PACEI50L	0.41 ± 0.07	1.31 ± 0.13	1.38 ± 0.06†

Cytotoxic potency is expressed as IC<sub>50</sub>, defined as the peptide concentration expected to reduce cell viability by 50% and calculated for each experiment by nonlinear regression of the experimental data to a four-parameter logistic curve. Data are mean ± SEM from triplicate experiments and were subjected to two-way ANOVA followed by Bonferroni post-tests.

\*Significantly different from the other two cell types,  $P < 0.05$ .

†Significantly different from PACEI32L,  $P < 0.01$ .

#### **4. DISCUSSION**

In this study we have designed and characterized a set of six novel heptapeptide sequences (Table 1) based on the previously identified PACEI32 and PACEI34 hexapeptides that showed ACE inhibitory effects both in vitro and ex vivo [4]. Our rational peptide designs were based on the recognition of similarities between C terminal residues of PACEI32/PACEI34 and angiotensin I, and combinatorialized the three alternative residues (F, H and L) between the two W residues at positions 3 and 6 in PACEI hexapeptides (see also Table 1). We have taken a comprehensive experimental approach to assess the in vitro ACE inhibitory effect using three enzyme substrates, the ex vivo inhibition of ACE-dependent vasoconstriction in isolated arteries, and the in vivo reduction of arterial blood pressure after either oral or intravenous administration to rats, as well as the in vitro potential toxicity in cultured cells. By doing so, the study allowed significant observations on the structure-activity relationships of new ACE inhibitory peptides, their antihypertensive effect, mechanism of action and cellular safety.

The in vitro test to assess the inhibitory effects of the six heptapeptides on ACE activity was carried out with three different substrates: the synthetic substrate HHL, and the natural substrates angiotensin I and bradykinin. Despite using the same heptapeptide concentration (20  $\mu$ M), residual ACE activities with HHL were always above 50% whereas with angiotensin I they were always below 50%, and no inhibition of bradykinin cleavage was found. Somatic ACE is an unusual enzyme which has two homologous domains with functional active sites, but with different biochemical properties including distinct substrate specificity and inhibitor affinity profiles [6]. The efficacy of ACE inhibitors is clearly substrate dependent in human ACE [18]. Our results point to substrate dependence in the inhibitory effects of the heptapeptides on porcine ACE activity, in contrast to similar inhibitory effects against HHL and angiotensin I cleavage previously reported for lactoferricin B-derived

peptides [4]. Interestingly, the heptapeptides did not show improved inhibitory effect against HHL cleavage when compared to lead hexapeptides [4] but the inhibition of angiotensin I cleavage was clearly improved. So, the heptapeptides showed higher ACE inhibitory effect with the natural substrate angiotensin I, and this is in favor of a potential antihypertensive effect.

With regard to the *in vitro* inhibitory potency of the heptapeptides against ACE activity with angiotensin I as substrate,  $IC_{50}$  values were in the low micromolar range. The majority of food protein derived ACE inhibitory peptides have moderate inhibitory potencies in the high micromolar range [7]. Our results are in line with the best inhibitory potencies reported for peptides derived from milk [20], cereal [7] and marine organisms [30]. All these *in vitro* inhibitory potencies are lower than those of ACE inhibitory drugs like captopril [19].

Our *ex vivo* experiments in rabbit isolated carotid artery showed that all the six heptapeptides inhibited angiotensin I-induced contraction with different efficacies ranging from 23% to 62% reduction of the contractile response. We have previously shown the captopril-sensitive, ACE-dependent nature of this angiotensin I-induced contraction [4]. Therefore, the present results add functional evidence for the ACE inhibitory effects of these heptapeptides in vascular tissue. However, the inhibitory effects of the different heptapeptides on ACE-dependent vasoconstriction did not correlate with the inhibitory effects observed *in vitro* on ACE activity with the natural substrate. Similar lacks of correlation have been recently reported for lactoferricin B-derived peptides [24] and casein hydrolysates [22]. Although this discrepancy deserves further research, a methodological detail could at least in part account for it. The *in vitro* test was carried out with porcine ACE, while rabbit arteries were used in the *ex vivo* test, and variations in the inhibition profiles of ACE from different species have been reported [28]. On the other hand, preincubation with PACEI50L, which was the most effective inhibitor of angiotensin I-induced contraction, did not inhibit angiotensin II-induced contraction, an ACE-independent

vasoconstriction in the rabbit carotid artery [4]. This suggests that the inhibitory effects of heptapeptides are not due to antagonism of AT1 receptors that mediate vasoconstriction induced by angiotensin II [3]. Finally, the lack of effect of PACEI50L on KCl-induced contraction rules out a possible nonspecific effect of peptides on vascular contractility.

Globally, the heptapeptides assayed in this study showed improved ACE inhibitory properties, both *in vitro* and *ex vivo*, when compared to the hexapeptides of reference from which they were derived [4]. We have recently reported the antihypertensive properties of lactoferricin B-derived peptides [24]. ACE inhibitory potencies in the low micromolar range shown by some of the heptapeptides were similar to those reported for lactoferricin B-derived peptides. However, the inhibitory effects on ACE-dependent vasoconstriction were higher in most heptapeptides when compared to lactoferricin B-derived peptides.

Oral administration of the hexapeptide PACEI32L previously reported as ACE inhibitor [4] and of two ACE inhibitory heptapeptides selected from this study (PACEI50L and PACEI52L) induced antihypertensive effects in SHR at doses devoid of hypotensive effects in normotensive WKY rats. The time course of the effects of peptides was different to that of captopril. Although one of the heptapeptides showed higher antihypertensive effect than captopril in the short term (1-2 h), blood pressure returned to baseline values 24 h after administration of the three peptides, at which time the effect of captopril was partially maintained. The antihypertensive effects of the hexapeptide PACEI32L and the heptapeptide PACEI50L, but not of the heptapeptide PACEI52L, were also observed after *i.v.* administration to SHR. Moreover, a dose-dependent effect was shown for the representative heptapeptide PACEI50L. In contrast to the oral effects lasting up to 3 h, the *i.v.* effects disappeared within minutes. As already noted by others [29], although *in vitro* potencies of hexa- and heptapeptides were much lower than that of captopril, the peptides showed higher *in vivo* antihypertensive effects than would be expected from their *in vitro* effects. In fact, fivefold lower doses of the

peptides produced blood pressure lowering effects similar to or even higher than those of captopril. It has been suggested that peptides have higher affinities for tissues and are more slowly eliminated than captopril [9].

Evidence for the stereoselective effects of heptapeptide PACEI50 was obtained from in vitro, ex vivo and in vivo experiments. When the D-amino acid enantiomeric counterpart was used, the potency against ACE activity was strongly reduced, the inhibitory effect on ACE-dependent vasoconstriction was missing at the concentration used, and the oral antihypertensive effect was not observed. In contrast, the antihypertensive effect after i.v. administration changed from transient (1-2 min) to long lasting (at least 1 h). We have previously reported similar in vitro and ex vivo effects for the L- and D-amino acid counterparts of the hexapeptide of reference PACEI32 [4]. In the present study, PACEI32D also lacked oral antihypertensive effect and showed long lasting i.v. antihypertensive effect. Different stereoselectivities have also been reported for tri- and tetralactopeptides at inhibiting ACE, although the antihypertensive effects were not studied [14].

Bioavailability has to be considered when ACE inhibitory effects are related with antihypertensive effects of bioactive peptides [29]. Some conclusions can be drawn from the comparison of effects according to the route of administration and stereoselectivity. In order to reduce blood pressure after oral administration, PACEI32L, PACEI50L and PACEI52L must remain at least in part intact after gastrointestinal digestion and intestinal absorption to reach the bloodstream, although the degradation to peptidic fragments retaining antihypertensive effects cannot be discarded, as recently reported for lactoferricin B-derived peptides [24]. Our results from i.v. administration suggest that once inside the circulation, the antihypertensive effect is rapidly reversed by blood peptidases, which is in line with acute transient antihypertensive effects described for i.v. food-derived peptides [16]. Although cotransport of protons and peptides is now recognised as a major route for intestinal absorption [17], D-amino acid peptides are poor substrates for transepithelial transporters [12], which

could explain their lack of oral effects in our assays. In contrast with the low ACE inhibitory effect of these peptides, they showed long-lasting antihypertensive effects after i.v. administration likely related with their resistance to blood peptidases [1]. In addition, all peptides used in this study have both termini protected, which could be of interest in retaining their *in vivo* effects.

Food-derived peptides are considered as generally safe because the parent proteins are present in the human diet, and specific toxicity tests have substantiated the safety of lactotripeptide consumption [2]. However, safety cannot be presumed for synthetic peptides. Our results show that cytotoxic potencies of the representative hexa- and heptapeptide assayed are much lower than their ACE inhibitory potencies. On the other hand, cytotoxic potencies of the present peptides are also lower than those reported for the food grade bacteriocin nisin, determined using the MTT assay and several cell types as well [13,27].

In conclusion, we have obtained novel rationally designed heptapeptides with improved ACE inhibitory properties when compared to lead hexapeptides. One selected hexapeptide (PACEI32L) and two heptapeptides (PACEI50L and PACE52L) show oral antihypertensive effects in SHRs, despite their susceptibility to peptidase attack in the bloodstream. Moreover, these peptides do not show hypotensive effects in normotensive rats and appear safe in cytotoxicity assays. The antihypertensive effects shown in SHRs were similar to those of commercial captopril, the gold standard for ACE inhibition. Future efforts will be directed to confirm the peptide safety and efficacy in daily intake experiments with SHRs and eventually in clinical trials with hypertensive subjects, and on the other hand to design new peptide sequences with improved ACE inhibitory effect, resistance to gastrointestinal digestion, intestinal absorption and resistance to blood peptidases, in order to optimize the antihypertensive effect. The peptidic nature of these potential antihypertensive drugs would allow their production through biotechnological processes.

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## **DISCUSIÓN**

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En esta tesis se ha caracterizado la actividad inhibidora de la enzima convertidora de la angiotensina I (ECA) y el efecto antihipertensivo de péptidos derivados de la lactoferrina bovina (LFb) y de péptidos no naturales diseñados *de novo*.

En relación con los péptidos derivados de LFb se han seguido dos estrategias. La primera de ellas ha consistido en caracterizar una serie de péptidos obtenidos a partir de deleciones y elongaciones de los extremos N-terminal y C-terminal de las secuencias de dos péptidos representativos de la lactoferrina bovina (LfcinB), LfcinB<sub>20-25</sub> (RRWQWR) y LfcinB<sub>17-31</sub> (FKCRRWQWRMKKLGA), cuya actividad inhibidora de la ECA fue demostrada por nuestro grupo de trabajo (Centeno y cols., 2006). La segunda estrategia ha consistido en la identificación de péptidos derivados de la LFb localizados en otras zonas de su secuencia, a partir de un hidrolizado generado con pepsina.

En lo referente a los péptidos diseñados *de novo*, se ha partido de dos hexapéptidos (PACEI32 y PACEI34) con capacidad inhibidora de la ECA previamente demostrada por nuestro grupo (Centeno y cols., 2006). A partir de éstos y, basándonos en los estudios estructurales que indican que son las posiciones C-terminal las importantes para la capacidad inhibidora de la ECA por parte de los péptidos (Cheung y cols., 1980; Li y cols., 2004; Wu y cols., 2006), se han diseñado una serie de heptapéptidos mediante combinaciones de residuos (Fenilalanina, Histidina y Leucina) en las posiciones 4, 5 y 6 de la secuencia peptídica.

Para determinar la actividad inhibidora de la ECA y el efecto antihipertensivo se han realizado ensayos *in vitro*, *ex vivo* e *in vivo*. Los ensayos *in vitro* han consistido en determinar los efectos inhibitorios por parte de los péptidos sobre la ECA utilizando diferentes sustratos artificiales y naturales. Por su parte, los ensayos funcionales *ex vivo*, usando segmentos arteriales aislados de conejo, nos han permitido estudiar los efectos inhibitorios sobre la vasoconstricción ECA-dependiente. Para estudiar el efecto antihipertensivo se han realizado

ensayos *in vivo* mediante la administración oral e intravenosa a ratas espontáneamente hipertensas (SHR) y a ratas normotensas Wistar-Kyoto (WKY). A su vez también se han realizado estudios *in vitro* con cultivos celulares para estudiar el potencial efecto tóxico de los péptidos no naturales y estudios de biodisponibilidad simulando la digestión gastrointestinal. Todo ello, ha permitido la caracterización de una serie de péptidos con efecto antihipertensivo, así como obtener información sobre la relación entre su secuencia y su bioactividad, sobre su biodisponibilidad, su seguridad, y los posibles mecanismos por los que ejercen su efecto fisiológico.

### **1. Estudios *in vitro* de inhibición enzimática**

Los estudios *in vitro* de inhibición enzimática están aceptados como el primer paso en la selección de péptidos con potencial efecto antihipertensivo que, posteriormente, serán evaluados en animales de experimentación, aunque como se ha demostrado en distintos trabajos y también en esta tesis no existe una correlación clara entre potencia inhibitoria *in vitro* y efecto antihipertensivo *in vivo*.

La inhibición de la ECA puede provocar tanto una disminución en la generación de angiotensina II (Ang II) a partir de angiotensina I (Ang I), como en la degradación de la bradiquinina. Por tanto, los ensayos de inhibición enzimática pueden llevarse a cabo utilizando cualquiera de los dos sustratos de la ECA, Ang I o bradiquinina. En general, se utilizan sustratos artificiales que mimetizan el extremo C-terminal de la Ang I, como el dipéptido sintético hipuril-L-histidil-L-leucina (HHL) o el tripéptido *o*-aminobenzoilglicil-*p*-nitrofenilalanilprolina (Abz-Gly-Phe(NO<sub>2</sub>)-Pro). En este trabajo, dependiendo del tipo de péptido caracterizado, se han utilizado, como sustratos de ECA, los dos sustratos artificiales mencionados, además de Ang I y bradiquinina, los dos sustratos fisiológicos de la enzima.



En estudios previos realizados por nuestro grupo (Centeno y cols., 2006) se demostró que, en función de que se utilizara HHL o Ang I como sustrato, la potencia inhibidora de los hexapéptidos PACEI variaba, siendo la inhibición mayor en el caso de emplearse el sustrato natural. Por el contrario, los dos péptidos derivados de LfcinB (LfcinB<sub>20-25</sub> y LfcinB<sub>17-31</sub>) mostraron la misma potencia inhibitoria independientemente del sustrato empleado. Una posible explicación a estos resultados sería el diferente mecanismo de inhibición para los hexapéptidos y los péptidos derivados de LfcinB. Teniendo en cuenta que el sustrato HHL mimetiza el dipéptido liberado por acción de ECA a partir de Ang I, los péptidos derivados de LfcinB interferirían con el reconocimiento, por parte de la enzima de esta zona. Sin embargo, los hexapéptidos PACEI, mucho más inhibitorios cuando se utiliza Ang I en lugar de HHL, además de interferir con esa zona tendrían otros efectos sobre el centro activo de la enzima.

En este trabajo hemos confirmado la dependencia del efecto inhibitorio de los heptapéptidos del sustrato de ECA empleado. A una concentración de 20  $\mu$ M, la actividad inhibidora de ECA utilizando HHL es siempre inferior a un 50%, mientras que con Ang I como sustrato, esta inhibición es superior al 50%. Resultados similares se han descrito para el captopril, cuya eficacia inhibitoria de la ECA humana varía en función del péptido empleado como sustrato (Michaud y cols. 1997). En nuestro caso, los resultados demuestran que la eficacia inhibitoria de los heptapéptidos sobre ECA porcina también es sustrato dependiente. Es de destacar que, con HHL, el efecto inhibitorio de los heptapéptidos no es mejor que el mostrado por los hexapéptidos parentales, lo que sí se ha observado en el caso de utilizarse Ang I. Este resultado, además de validar el criterio de diseño de los heptapéptidos, favorecería su efecto antihipertensivo *in vivo*. Asimismo, hemos confirmado que los heptapéptidos no tienen ningún efecto inhibitorio sobre ECA cuando se utiliza la bradiquinina como sustrato, tal y como habíamos descrito para los hexapéptidos (Centeno y cols., 2006). Estos resultados indican que los PACEIs interaccionan con el sitio de unión de Ang I. En concordancia con estos datos, se ha descrito que el

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valor de  $IC_{50}$  de péptidos inhibidores derivados de proteínas lácteas cuando se utiliza un sustrato similar a la Ang I es mucho menor que el valor de  $IC_{50}$  cuando se utiliza un sustrato similar a la bradiquinina, lo que se interpreta como que los inhibidores interfieren preferiblemente con el metabolismo de la angiotensina. En estudios en ratones se ha demostrado que la inhibición selectiva del dominio N o del dominio C de ECA impide la conversión de Ang I en Ang II, mientras que para impedir la hidrólisis de bradiquinina sería necesaria la interacción del inhibidor con los dos dominios (Georgiadis y cols., 2003). Además, algunos autores han señalado que los inhibidores de ECA actúan a nivel de los receptores de la bradiquinina en lugar de bloqueando su hidrólisis (Tom y cols., 2003).

La potencia inhibitoria, utilizando Ang I como sustrato, tanto de los heptapéptidos PACEI como de la serie de péptidos derivados de LfcinB es muy similar, ya que los valores de  $IC_{50}$  oscilan entre 3.0 y 16.4  $\mu\text{M}$  para los primeros y entre 2.3 y 32.0  $\mu\text{M}$  para los segundos. En el caso de los péptidos derivados de LFb, cuyo  $IC_{50}$  varía entre 0.47 (LIWKL) y 105.3  $\mu\text{M}$  (LNNSRAP), los ensayos se han realizado utilizando Abz-Gly-Phe(NO<sub>2</sub>)-Pro como sustrato, ya que esta metodología permite, además de procesar un alto número de muestras, evaluar sin interferencias muestras peptídicas complejas como el hidrolizado de LF y sus fracciones. Valores de  $IC_{50}$  en el rango micromolar, como los descritos en esta memoria, son los usuales para péptidos antihipertensivos derivados de proteínas lácteas (ver Introducción Tabla 2; Hernández-Ledesma y cols., 2011), de vegetales y de otras fuentes proteicas (Martínez-Maqueda y cols., 2012). Es interesante destacar que algunos de los péptidos caracterizados en este trabajo son al menos igual de potentes que los lactotripéptidos VPP ( $IC_{50} = 9 \mu\text{M}$ ) e IPP ( $IC_{50} = 5 \mu\text{M}$ ) cuyo efecto antihipertensivo en humanos ha sido demostrado en diferentes estudios (Nakamura y cols., 1995a y b). Tal y como se comentó en la introducción de esta memoria, hasta la fecha sólo se ha caracterizado un péptido antihipertensivo derivado de LFb, de secuencia LRPVAA, con un valor de  $IC_{50}$  de 4.14  $\mu\text{M}$  (Lee y cols., 2006). Por lo que se refiere al hidrolizado de

LFb de tamaño molecular inferior a 3 KDa, su potencia inhibitoria es comparable a la descrita para hidrolizados similares de caseína (5.68 µg/ml; Contreras y cols., 2009) y 60 veces superior a la del hidrolizado no ultrafiltrado (950 µg/ml).

Finalmente, resulta interesante destacar que, al igual que todos los inhibidores de la ECA derivados de proteínas alimentarias, los péptidos e hidrolizados aquí caracterizados presentan una potencia inhibitoria muy inferior a la de los fármacos antihipertensivos. A modo de ejemplo remarcar que el IC<sub>50</sub> determinado para el captopril es de 0.022 µM (Ondetti y cols., 1977).

## **2. Relación entre la secuencia peptídica y la potencia inhibitoria *in vitro* sobre la ECA**

Los estudios *in vitro* de inhibición enzimática permiten establecer una relación entre secuencia peptídica y potencia inhibitoria. Sin embargo, y a pesar de los numerosos estudios que han abordado esta relación, los resultados no son totalmente concluyentes, ya que además de la secuencia aminoacídica, también es importante la longitud del péptido y su potencial electrostático (Li y cols., 2004; De Leo y cols., 2009). En general, los péptidos inhibidores de ECA derivados de proteínas alimentarias comparten ciertas características estructurales como su longitud (2-15 residuos de aminoácidos) y la secuencia del tripéptido C-terminal que, usualmente, contiene residuos de aminoácidos hidrofóbicos (tirosina, fenilalanina y triptófano) junto con residuos de prolina, lisina o arginina (Murray y FitzGerald, 2007). En concreto, se ha descrito que la presencia de un residuo de prolina en antepenúltima posición favorece la unión del péptido inhibidor al centro activo de ECA (Rohrbach y cols., 1981), y también la importancia de un residuo de leucina en el extremo C-terminal para aumentar la potencia inhibitoria de los péptidos (Gómez-Ruiz y cols., 2004).

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La mayoría de los péptidos evaluados en este trabajo cumplen alguna de estas características estructurales. Los péptidos derivados de LfcinB presentan en el extremo C-terminal un residuo de arginina (LfcinB<sub>20-25</sub>, LfcinB<sub>19-25</sub>, LfcinB<sub>18-25</sub> y LfcinB<sub>17-25</sub>), triptófano (LfcinB<sub>17-24</sub> y LfcinB<sub>17-22</sub>) o prolina (LfcinB<sub>17-32</sub>). La única excepción es el péptido LfcinB<sub>17-31</sub> en cuyo extremo C-terminal hay un residuo de alanina. Por lo que respecta a los PACEIs, todos ellos presentan un residuo de triptófano en el extremo C-terminal mientras que los tres péptidos derivados de LFb presentan un residuo de leucina o prolina. En el caso del péptido RPYL, es de destacar también la presencia de un residuo de prolina en la antepenúltima posición. Todos ellos presentan capacidad inhibitoria de la ECA en mayor o menor medida, pero siempre dentro del rango micromolar como ya se ha comentado. También es interesante destacar que algunos péptidos derivados de LFb evaluados en este trabajo y que presentaban también algunas de estas características estructurales, como por ejemplo LHQQAL, YLGSRY o AVAKF, no presentan capacidad de inhibición de la enzima.

En este trabajo hemos evaluado la potencia inhibitoria de una serie de péptidos derivados de diferentes elongaciones a partir de los extremos amino y carboxilo terminal de LfcinB<sub>20-25</sub> (RRWQWR), lo que ha permitido extraer ciertas conclusiones sobre el efecto de la secuencia aminoacídica en la potencia inhibitoria. En primer lugar, nuestros resultados subrayan la influencia del extremo N-terminal en la inhibición, dado que se observa que los péptidos LfcinB<sub>19-25</sub> (CRRWQWR; IC<sub>50</sub> = 2.3 μM), LfcinB<sub>18-25</sub> (KCRRWQWR; IC<sub>50</sub> = 5.8 μM) y LfcinB<sub>17-25</sub> (FKCRRWQWR; IC<sub>50</sub> = 2.9 μM), derivados de elongaciones a partir del extremo amino terminal de LfcinB<sub>20-25</sub>, muestran mayor potencia inhibitoria que el péptido parental (IC<sub>50</sub> = 32 μM). Es de destacar que, en general, los diferentes estudios publicados remarcan la importancia del tripéptido C-terminal en la inhibición de ECA, y que incluso estudios de modelización de péptidos han mostrado que no existe relación entre los residuos del extremo N-terminal y la potencia inhibitoria (Wu y cols., 2006), aunque estos mismos autores señalaban que

en péptidos con más de seis residuos aminoacídicos, como los tres derivados de LfcinB comentados, deben ser tenidos en cuenta los posibles efectos estéricos. Otra de las conclusiones que se puede extraer de este trabajo confirma la importancia del residuo de prolina en el extremo C-terminal de la secuencia (Li y cols., 2004), dado que la potencia inhibitoria de LfcinB<sub>17-32</sub> (FKCRRWQWRMCKLGA; IC<sub>50</sub> = 11 µM) es superior a la de LfcinB<sub>17-31</sub> (FKCRRWQWRMCKLGA; IC<sub>50</sub> = 25.5 µM). Además, la diferencia entre las potencias inhibitorias de LfcinB<sub>17-25</sub> (FKCRRWQWR; IC<sub>50</sub> = 2.9 µM) y LfcinB<sub>17-24</sub> (FKCRRWQW; IC<sub>50</sub> = 10.5 µM) refuerza la importancia del residuo de arginina en la posición C-terminal, a cuya carga positiva se ha atribuido la potencia inhibitoria de diversos péptidos derivados de caseína (FitzGerald y Meisel, 2000).

Los heptapéptidos PACEI han sido diseñados basándose en las secuencias de los hexapéptidos previamente identificados PACEI32 (RKWHFW; IC<sub>50</sub> = 10.7 µM) y PACEI34 (RKWLFW; IC<sub>50</sub> = 8.1 µM) con actividad inhibitoria *in vitro* de la ECA (Centeno y cols., 2006). El diseño de los heptapéptidos se ha basado en la similitud de los residuos del extremo C-terminal de PACEI32 y PACEI34 con los de la Ang I (DRVYIHPFHL), y la combinatorialización de los residuos de fenilalanina, histidina y leucina entre los dos residuos de triptófano en las posiciones 3 y 6 de los hexapéptidos. Excepto en el caso de PACEI48 (RKWFHLW; IC<sub>50</sub> = 16.4 µM), el resto de los heptapéptidos han mostrado valores de IC<sub>50</sub> por debajo de 10 µM, aunque sin diferencias significativas entre ellos. Curiosamente, el péptido menos potente PACEI48 contiene la secuencia FHL en el mismo orden que la Ang I. La pareja de péptidos PACEI48 (RKWFHLW) y PACEI49 (RKWFLHW) muestran permutaciones de los residuos en las posiciones 5 y 6 lo que conlleva un aumento de la potencia inhibitoria de PACEI49 (IC<sub>50</sub> = 5.9 µM). También se traduce en un aumento de potencia las permutaciones de los residuos en las posiciones 4 y 5 de PACEI48 y PACEI50 (RKWHFLW; IC<sub>50</sub> = 8.2 µM). Sin embargo, las permutaciones entre PACEI49 y PACEI52 (RKWLFHW) o entre PACEI51 (RKWHFLW) y

PACEI53 (RKWLHFW) no han producido ningún efecto significativo sobre la potencia inhibitoria.

En su conjunto, estos resultados demuestran los efectos inhibitorios *in vitro* sobre ECA de péptidos derivados de distintas zonas de LFb y de los heptapéptidos obtenidos por diseño racional, y además confirman resultados previos sobre la influencia que tienen cambios de un sólo aminoácido en la actividad biológica de una determinada secuencia. También ponen de manifiesto las complejas relaciones secuencia-actividad en péptidos de más de tres residuos aminoacídicos. Asimismo, como se comenta más adelante, muchas de las conclusiones obtenidas con los ensayos *in vitro* no se sustentan con los resultados de los ensayos *ex vivo* e *in vivo*.

### **3. Estudios *ex vivo*: efectos sobre la vasoconstricción ECA-dependiente**

En ocasiones el efecto inhibitor de la actividad ECA demostrado *in vitro* para un péptido bioactivo o para un hidrolizado peptídico no se traduce en un efecto antihipertensor observado *in vivo* en un modelo animal de hipertensión. Los estudios *ex vivo* con arterias aisladas en baño de órganos registran efectos vasoactivos y de ese modo aportan evidencia funcional de la capacidad de los péptidos inhibidores de la ECA para inhibir la vasoconstricción ECA-dependiente. Con esta evidencia funcional se pretende incrementar la probabilidad de que los efectos *in vitro* se correlacionen con los efectos *in vivo*.

La validez de los ensayos *ex vivo* ha sido demostrada en estudios previos realizados por nuestro grupo (Centeno y cols., 2006) habiéndose comprobado que la Ang I induce vasoconstricción dependiente de la ECA en arteria carótida de conejo, ya que el fármaco inhibidor de la ECA captopril inhibe de manera concentración-dependiente la contracciones inducidas por la Ang I pero no las inducidas por la Ang II.

Los ensayos *ex vivo* realizados con los péptidos derivados de LfcinB han mostrado que la mayoría de ellos tienen efectos inhibidores sobre la vasoconstricción ECA-dependiente. Los valores de inhibición de vasoconstricción oscilan entre el 18% para LfcinB<sub>17-24</sub> y el 29% para LfcinB<sub>20-25</sub>. Cuatro de los péptidos superan, aunque no muy ostensiblemente, el efecto inhibidor descrito anteriormente para LfcinB<sub>17-31</sub> (Centeno y cols., 2006). Excepcionalmente, LfcinB<sub>19-25</sub> es el único péptido que no ha mostrado efecto inhibidor de la vasoconstricción dependiente de la ECA, a pesar de que *in vitro* posee una elevada potencia como inhibidor de la ECA (IC<sub>50</sub>=2.3 µM). En general, no hay correlación entre la potencia *in vitro* de los péptidos derivados de LfcinB y su efecto inhibidor *ex vivo* sobre la vasoconstricción ECA-dependiente, a pesar de utilizar Ang I como sustrato en ambos tipos de experimentos.

Por otra parte, los seis heptapéptidos PACEI muestran efectos inhibidores de la vasoconstricción ECA-dependiente en los ensayos *ex vivo*, con diferentes grados de reducción de la vasoconstricción respecto al control oscilando entre el 23% del menos potente (PACEI53L) y el 62% del más potente (PACEI50L). Tampoco hay correlación entre la potencia *in vitro* de los seis heptapéptidos y su efecto inhibidor *ex vivo* sobre la vasoconstricción ECA-dependiente. Globalmente, los heptapéptidos muestran mejores efectos inhibidores de la ECA, tanto *in vitro* como *ex vivo*, que los dos hexapéptidos de referencia a partir de los que se derivaron (Centeno y cols., 2006). Además, los heptapéptidos también muestran mejores efectos inhibidores *ex vivo* que los péptidos derivados de LfcinB, a pesar de mostrar potencias inhibidoras *in vitro* similares en el rango micromolar.

El hidrolizado con pepsina LFH tiene efectos inhibidores de la vasoconstricción con Ang I, tanto reduciendo la magnitud de la contracción como enlenteciéndola, lo cual evidencia *ex vivo* la capacidad del hidrolizado para inhibir la actividad ECA *in vitro*. Ni la LF no hidrolizada ni la pepsina por separado producen inhibición de la vasoconstricción ECA-dependiente, lo cual confirma que es necesaria la hidrólisis de la proteína

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LF para producir péptidos bioactivos responsables de la inhibición de la actividad ECA tanto *in vitro* como *ex vivo*.

Finalmente, los tres péptidos del hidrolizado ultrafiltrado LFH<3KDa seleccionados por su efecto inhibitor de la actividad ECA *in vitro* muestran valores de reducción de la vasoconstricción ECA-dependiente del 14% para RPYL, del 23% para LIWKL y del 10% para LNNSRAP, siendo la inhibición no significativa en el último caso. Cabe resaltar el relativamente similar efecto inhibitor *ex vivo* de RPYL y LIWKL a pesar de una importante diferencia en aproximadamente dos órdenes de magnitud de sus potencias inhibitoras de la ECA *in vitro*, así como la falta de efecto *ex vivo* de LNNSRAP a pesar de su efecto inhibitor *in vitro*. En cualquier caso, los efectos inhibitor *ex vivo* de los péptidos RPYL y LIWKL son en términos generales similares a los mostrados por los péptidos derivados de LfcinB y menores que los mostrados por los heptapéptidos PACEI.

Los resultados obtenidos en los ensayos *ex vivo*, tanto con los péptidos derivados de LfcinB como con los heptapéptidos PACEI y con los péptidos derivados del hidrolizado LFH<3KDa, muestran una falta de correlación con los resultados obtenidos en los ensayos *in vitro*, lo cual indica la compleja relación existente entre la secuencia de los péptidos y su actividad biológica. Por otra parte, esta falta de correlación observada entre los efectos inhibitor sobre la vasoconstricción dependiente de la ECA y los efectos inhibitor sobre la actividad de la ECA también ha sido observada por otros autores en hidrolizados de caseína (Rousseau-Ralliard y cols., 2010) y podría deberse, al menos en parte, a detalles metodológicos como el uso de diferentes especies animales en cada uno de los ensayos: la ECA utilizada para los ensayos *in vitro* es de origen porcino, mientras que los ensayos *ex vivo* utilizan arterias carótidas de conejo. Se han descrito variaciones en el perfil de inhibición de la ECA dependiendo de la especie animal de origen (Vazeux y cols., 2001).



De todos los péptidos ensayados, el heptapéptido PACEI50L es el más efectivo inhibiendo la vasoconstricción ECA-dependiente *ex vivo*. Por eso se ha elegido este péptido para identificar o descartar mecanismos adicionales a la inhibición de la ECA en el efecto inhibitor de la vasoconstricción. En primer lugar, se ha comprobado el efecto de PACEI50L sobre la vasoconstricción inducida por Ang II, la cual es insensible al captopril y por tanto ECA-independiente (Centeno y cols., 2006). PACEI50L no muestra efecto inhibitor de la vasoconstricción con Ang II, sugiriendo que el mecanismo de acción del péptido no es debido a su actuación como antagonista de los receptores de angiotensina tipo I (RAT<sub>1</sub>) que median la respuesta a la Ang II (Carey y Siragy, 2003), sino que se limita a actuar a nivel de la ECA. Por otra parte, la falta de efecto inhibitor de PACEI50L sobre la vasoconstricción inducida por KCl descarta un posible efecto no específico del péptido sobre la contractibilidad vascular.

También se ha realizado el ensayo utilizando Ang II con el hidrolizado LFH. En este caso, la respuesta vasoconstrictora a Ang II en presencia de LFH no solamente no resulta inhibida, lo cual descarta el antagonismo de los RAT<sub>1</sub>, sino que resulta en cambio ligera pero significativamente aumentada. La Ang II es degradada en segundos por varias peptidasas, colectivamente denominadas angiotensinasas, que forman diferentes fragmentos inactivos como la des-aspartil-angiotensina-II (angiotensina III), angiotensina (1-7) y angiotensina (3-8) (Carey y Siragy, 2003). La potenciación de la vasoconstricción inducida por Ang II sugiere que el hidrolizado LFH, además del efecto inhibitor sobre la ECA, puede tener efecto inhibitor sobre la degradación de la Ang II vasoconstrictora que produce péptidos no-vasoactivos.

#### **4. Estudios *in vivo*: efectos antihipertensivos**

La falta general de correlación entre los efectos inhibidores de la actividad ECA *in vitro* y los efectos inhibidores de la vasoconstricción ECA-dependiente *ex vivo* motiva la necesidad de estudiar *in vivo* los efectos antihipertensivos de los péptidos bioactivos en un modelo animal de hipertensión como la rata espontáneamente hipertensa (SHR). Los péptidos a estudiar *in vivo* se seleccionan en función de la mayor potencia *in vitro*, de la mayor eficacia *ex vivo*, o de ambas cuando coincidan las dos circunstancias.

En numerosos estudios precedentes sobre el efecto antihipertensivo de péptidos inhibidores de la ECA que proceden de proteínas alimentarias, administrados a ratas hipertensas por vía oral, se obtuvieron valores de reducción de presión sistólica entre -0.6 y -80.0 mmHg (Murray y FitzGerald, 2007).

En nuestro caso, de los péptidos derivados de LfcinB ensayados, solamente LfcinB<sub>20-25</sub> muestra efecto antihipertensivo (-16.7 mmHg) una hora después de administrarse a ratas SHR, que en términos relativos supone una reducción del 7.7% respecto a la presión basal. Estos resultados *in vivo* contrastan con los resultados de los experimentos *ex vivo*, que muestran la capacidad de seis péptidos derivados de LfcinB para inhibir la vasosonstricción ECA-dependiente, tres de ellos con valores de inhibición similares al de LfcinB<sub>20-25</sub>.

En cuanto a los efectos *in vivo* de los péptidos PACEI, el hexapéptido de referencia PACEI32L muestra un efecto antihipertensivo de -24.6 mmHg (reducción del 11.6%), mientras que el heptapéptido PACEI50L muestra un efecto antihipertensivo de -36.3 mmHg (17.2%) y el heptapéptido PACEI52L un efecto de -18.6 mmHg (9.1%). En cuanto al curso de estos efectos antihipertensivos, el pico de reducción de presión se produce durante la primera hora post-administración para PACEI32L y PACEI50L, y durante la segunda hora para PACEI52L. En cualquier caso, el efecto antihipertensivo es transitorio y se mantiene como mucho hasta

las tres horas en el caso de PACEI50L. Para estos péptidos PACEI, el mayor efecto inhibitor de vasoconstricción ECA-dependiente mostrado por el heptapéptido PACEI50L *ex vivo* se traduce en el mayor efecto antihipertensivo *in vivo*.

El hidrolizado con pepsina LFH no tiene efecto antihipertensivo en ratas SHR, a pesar de haber mostrado efectos inhibidores tanto de la actividad ECA como de la vasoconstricción ECA-dependiente. En cambio, el hidrolizado ultrafiltrado LFH<3KDa sí que muestra efecto antihipertensivo (-15.4 mmHg), lo cual indica que el efecto antihipertensivo debe atribuirse principalmente al componente peptídico del hidrolizado con peso molecular por debajo de 3KDa, como se ha propuesto para otros hidrolizados (Mullaly y cols., 1997). De hecho, la potencia del hidrolizado LFH<3KDa para inhibir la actividad ECA *in vitro* ( $IC_{50} = 14.3 \mu\text{g/mL}$ ) es 60 veces mayor que la potencia del hidrolizado LFH crudo, antes de ultrafiltrar ( $IC_{50} = 950 \mu\text{g/ml}$ ) (Ruiz-Giménez y cols., 2007).

Los tres péptidos identificados en el hidrolizado LFH<3KDa y seleccionados por su efecto inhibitor de la ECA muestran efecto antihipertensivo al administrarse oralmente a ratas SHR. RPYL induce una reducción en la presión arterial de -18.9 mmHg, lo que equivale al 9.3% de la presión basal, mientras que LIWKL induce una reducción de -25.3 mmHg (12.5%) y LNNSRAP una reducción de -15.3 mmHg (7.6%). Estos tres péptidos identificados en el hidrolizado LFH<3KDa se añaden a LfcinB<sub>20-25</sub> (RRWQWR) como péptidos derivados de LFb con propiedades antihipertensivas tras su administración oral. Previamente, se ha descrito LRPVAA como péptido inhibitor de la ECA aislado también de un hidrolizado con pepsina de LFb, con efecto antihipertensivo tras su administración intravenosa a ratas SHR (Lee y cols., 2006). Ni LfcinB<sub>20-25</sub> (RRWQWR), ni LRPVAA se han identificado en el hidrolizado LFH<3KDa obtenido en el presente estudio.

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De entre todos los péptidos e hidrolizados peptídicos con efecto antihipertensivo comprobados en este estudio, algunos merecen especial mención por diferentes razones. El péptido LfcinB<sub>20-25</sub>, por ser el primer péptido bioactivo con efecto antihipertensivo tras administración oral derivado de la lactoferrina, en concreto de su núcleo funcional lactoferrina. También destaca el heptapéptido PACEI50L por ser el que presenta mayor efecto antihipertensor entre todos los ensayados, superando incluso al efecto antihipertensor del captopril incluido en el estudio como control positivo. Por último, cabe resaltar los efectos antihipertensivos del hidrolizado LFH<3KDa y de su péptido LIWKL, que se mantienen tras 24 horas de su administración, como ocurre con el captopril, a pesar de administrarse de manera aguda en dosis única. En contraste con LFH<3KDa y de su péptido aislado LIWKL, los efectos antihipertensivos descritos para los hidrolizados <3KDa obtenidos con pepsina a partir de caseína bovina o clara de huevo, así como los efectos de la mayoría de los péptidos aislados de dichos hidrolizados, son transitorios y revierten a las 24 horas desde su administración (Miguel y cols., 2009; Contreras y cols., 2009; Miguel y cols., 2005).

En nuestros ensayos *in vivo*, la administración oral de los péptidos analizados produce mayor efecto antihipertensivo de lo que se esperaría por el efecto inhibidor de ECA que se observa *in vitro*. Así, aunque el captopril posee una capacidad inhibidora 1000 veces superior a los péptidos inhibidores de la ECA en los ensayos *in vitro*, cuando se comparan los resultados *in vivo* no existen tales diferencias. Resultados similares han sido observados por otros autores (Li y cols., 2004; Suetsuna y cols., 1998), concluyéndose que en comparación con los fármacos antihipertensivos como el captopril, los péptidos inhibidores de la ECA derivados de alimentos poseen en general mayor efecto antihipertensivo *in vivo* respecto al efecto que se observa *in vitro*. Es posible que la mayor afinidad tisular y la eliminación más lenta de los péptidos bioactivos sean algunos de los mecanismos que explican estas diferencias con el captopril (Fujita y cols., 1999). También se propone la posibilidad de que estos

péptidos, a diferencia del captopril, actúen simultáneamente a nivel de la inhibición de la ECA y a través de otros mecanismos alternativos que influyan sobre la presión arterial.

Aunque no se ha comprobado con todos los péptidos antihipertensivos utilizados en este estudio, los ensayos mediante administración oral realizados con PACEI32L, PACEI50L y PACEI52L, muestran una falta de efecto sobre la presión arterial cuando fueron administrados a ratas normotensas WKY. En el caso de que este efecto diferencial se mantuviera en humanos, sería un valor añadido para estos péptidos ya que mostrarían efectos sobre pacientes hipertensos sin que tuvieran efectos sobre sujetos normotensos.

## **5. Biodisponibilidad**

Los efectos fisiológicos de los péptidos bioactivos dependen de que puedan llegar en forma activa a su órgano diana. Esto implica que un péptido, tras su administración oral, debe ser resistente a las proteasas gastrointestinales y a las peptidasas del borde en cepillo de las células intestinales. A continuación debe ser absorbido en el epitelio intestinal y, una vez en el torrente sanguíneo, el péptido debe resistir la actividad enzimática de las proteasas sanguíneas antes de alcanzar los órganos diana (Vermeirssen y cols., 2004; Ricci y cols., 2010).

La resistencia de los péptidos antihipertensivos derivados de proteínas alimentarias a estos posibles procesos de degradación, se suelen abordar mediante experimentos *in vitro* de simulación gastrointestinal. En este tipo de ensayos se realiza una hidrólisis secuencial con pepsina y extractos pancreáticos, para a continuación realizar estudios *in vitro* de absorción intestinal utilizando la línea celular Caco-2. Estos estudios se completan con ensayos de estabilidad frente a proteasas plasmáticas.

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La escasa biodisponibilidad de los péptidos antihipertensivos tras su administración oral, suele ser una de las razones aducidas para explicar las discrepancias entre la capacidad inhibidora de la ECA *in vitro* y la capacidad antihipertensiva *in vivo*. Por contra, también debe tenerse en cuenta que la degradación de los péptidos puede originar secuencias peptídicas con mayor potencia inhibitoria de la ECA y mayor efecto antihipertensivo (FitzGerald y cols., 2004). Aunque en un principio se pensaba que los péptidos eran rápidamente metabolizados a sus aminoácidos correspondientes, distintos estudios de biodisponibilidad han demostrado que algunos péptidos antihipertensivos son resistentes a los procesos fisiológicos de digestión y que también son absorbidos por el epitelio intestinal, alcanzando el torrente sanguíneo. Este es el caso de los lactotripéptidos VPP e IPP (Ohsawa y cols, 2008; Foltz y cols., 2008), e incluso de péptidos de mayor longitud ricos en prolina como el péptido antihipertensivo derivado de caseína HLPLP (Quirós y cols., 2008). Además, los tres secuencias han sido detectadas en plasma humano tras su administración oral (van Platerink y cols., 2006; Foltz y cols., 2007).

En el caso de los péptidos derivados de LfcinB, sólo uno de ellos, LfcinB<sub>20-25</sub>, ha mostrado efecto antihipertensivo en ratas SHR, a pesar de que todos ellos habían mostrado capacidad de inhibición *in vitro* de la ECA y, a excepción de LfcinB<sub>19-25</sub>, también efecto sobre la vasoconstricción dependiente de ECA. Cuando han sido sometidos a degradación gastrointestinal, todos ellos se han hidrolizado en mayor o menor medida, destacando la hidrólisis completa sufrida por LfcinB<sub>20-25</sub>. Estos resultados sugieren que posiblemente sus fragmentos de degradación, identificados como WQ y RWQ, serían los responsables del efecto antihipertensivo observado en las ratas. De hecho, WQ también ha mostrado efecto antihipertensivo *in vivo*, aunque inferior al causado por el hexapéptido, lo que apoyaría esta hipótesis. Sin embargo, hay que tener en cuenta que los ensayos de digestión gastrointestinal simulan las condiciones fisiológicas,

pero pueden no reflejar de forma real el proceso de digestión. Por tanto, no podemos descartar que LfcinB<sub>20-25</sub> en su forma intacta pudiera ser el responsable del efecto antihipertensivo, o que se debiera a un efecto sinérgico entre el hexapéptido y sus fragmentos de degradación. Estudios posteriores llevados a cabo por nuestro grupo de trabajo con la línea celular Caco-2, han demostrado que LfcinB<sub>20-25</sub> no es absorbido, en forma intacta, a través del epitelio intestinal, mientras que si se detecta transporte del dipéptido (Fernández-Musoles y cols, 2013). Estos resultados vuelven a señalar al dipéptido como posible responsable del efecto antihipertensivo, aunque serían necesarios más estudios para poder confirmarlo.

También en el caso de los péptidos derivados de LF, se observa degradación tras la digestión gastrointestinal, y en el caso del péptido con mayor efecto antihipertensivo *in vivo* (LIWKL), la hidrólisis resulta ser total. Al igual que en el caso de LfcinB<sub>20-25</sub>, los resultados obtenidos con LIWKL vuelven a señalar a los fragmentos de degradación, identificados como IW, LIW y IWK, como posibles responsables del efecto antihipertensivo en ratas. Aunque no se han abordado estudios *in vivo* con los tres fragmentos, conviene señalar que la secuencia IW fue descrita en los estudios pioneros de Cheung y cols. (1980) como uno de los dipéptidos con mayor potencia inhibitoria de ECA. Sin embargo, se requieren más estudios para poder asignar el efecto antihipertensivo a uno de los tres fragmentos, a la secuencia intacta o bien a un efecto aditivo o sinérgico

En resumen, nuestros resultados indican que los péptidos derivados de distintas zonas de LFb son altamente susceptibles de degradación por proteasas del tracto gastrointestinal, si bien en las condiciones ensayadas no se observa degradación por parte de la pepsina. Además, la degradación es independiente de que presenten los extremos N- y C-terminal protegidos, como es el caso de los derivados de LfcinB, por lo que la protección de los extremos, en las condiciones *in vitro*

evaluadas, no confiere mayor estabilidad frente a las proteasas gastrointestinales.

## **6. Mecanismos de acción**

Los mecanismos de control de la presión arterial son diversos e interrelacionados entre sí, siendo probablemente el sistema renina-angiotensina (RAS) el mecanismo humoral más importante. Dentro del RAS, la ECA es la enzima que hidroliza al péptido inactivo Ang I formando el péptido vasoconstrictor Ang II, el cual durante su permanencia en la sangre actúa a través de los  $RAT_1$  situados en el músculo liso produciendo vasoconstricción de las arteriolas, aumento de la resistencia e incremento de la presión arterial. Los experimentos *in vitro* y *ex vivo* de este estudio se han llevado cabo para identificar la inhibición de la actividad ECA como mecanismo de acción implicado en los efectos antihipertensivos de los péptidos bioactivos ensayados.

Los resultados obtenidos con los ensayos *in vitro* y *ex vivo*, han mostrado que la inhibición de la actividad ECA es el mecanismo de acción responsable, al menos en parte, de los efectos antihipertensivos del péptido derivado de lactoferrina LfcinB<sub>20-25</sub>, del hexapéptido PACEI32L, de los heptapéptidos PACEI50L y PACEI52L, así como del hidrolizado de lactoferrina LFH<3KDa y de sus péptidos RPYL y LIWKL. La inhibición de la actividad ECA es el mecanismo de acción generalmente propuesto para los péptidos antihipertensivos derivados de proteínas alimentarias (Vermeirssen y cols., 2004). Otros péptidos derivados de lactoferrina, así como el hidrolizado crudo LFH sin ultrafiltrar, no muestran efectos antihipertensivos a pesar de inhibir la actividad ECA, lo cual puede deberse a falta de biodisponibilidad, como se ha discutido en el apartado anterior.



Por el contrario, también se ha comprobado que algunos péptidos estudiados presentan actividad antihipertensiva cuando son administrados por vía oral pero no muestran efecto inhibitor de la actividad ECA. Por un lado, el dipéptido WQ, fragmento de degradación del péptido LfcinB<sub>20-25</sub> obtenido tras su digestión simulada *in vitro*, el cual muestra efecto antihipertensivo a pesar de no inhibir la vasoconstricción ECA-dependiente. Por otro lado, el heptapéptido LNNSRAP, identificado en el hidrolizado LFH<3KDa, muestra efecto antihipertensivo a pesar de inhibir con muy baja potencia la actividad ECA *in vitro* y no inhibir la vasoconstricción ECA-dependiente. Estas discrepancias entre los efectos *in vivo* y los efectos *in vitro* o *ex vivo* sugieren la intervención de un mecanismo de acción antihipertensivo diferente a la inhibición de la actividad ECA. Se ha descrito por ejemplo que la liberación de sustancias vasodilatadoras como prostaciclina, NO o CO podrían también contribuir a los efectos antihipertensivos de varios péptidos inhibidores de ECA (De Leo y cols., 2009). Recientemente se ha sugerido la argininsuccinato sintetasa, una importante enzima en el ciclo citrulina-NO que supone un paso limitante en la síntesis de NO, como una diana funcional para un péptido antihipertensivo obtenido de veneno de serpiente (Guerreiro y cols., 2009). Por último, la modulación de la endotelina-1 y un mecanismo a través de receptores opioides pueden intervenir en los efectos antihipertensivos de péptidos lácteos como la lactokinina (Maes y cols., 2004) y  $\alpha$ -lactorfina (Nurminen y cols., 2000), respectivamente. Estudios posteriores de nuestro grupo han demostrado que algunos péptidos derivados de LfcinB son inhibidores duales de vasopeptidasas (enzimas conversores de angiotensina y endotelina) (Fernandez-Musoles y cols., 2010).

## 7. Estereoselectividad

Este estudio, a través de los experimentos *in vitro*, *ex vivo* e *in vivo*, nos ha permitido establecer ciertas conclusiones sobre el efecto de la estereoselectividad de los péptidos sobre el efecto antihipertensivo. Los péptidos D son más estables y menos susceptibles a la degradación proteolítica que los sintetizados con aminoácidos L (Miller y cols., 1995), por lo que su empleo podría ser relevante para aplicaciones específicas que requieran, como en el caso del control de la hipertensión, que el péptido activo llegue intacto a su diana fisiológica. Sin embargo, la utilización de péptidos D tiene como principal inconveniente su elevado coste, debido a que únicamente pueden ser obtenidos por síntesis química.

Este trabajo nos permite comparar el efecto antihipertensivo de PACEI50 sintetizado con D- y L-aminoácidos. Cuando se evalúa PACEI50D, su potencia inhibitoria se reduce de forma notable (PACEI50D,  $IC_{50} = 45.7 \mu\text{M}$ ; PACEI50L,  $IC_{50} = 8.2 \mu\text{M}$ ), se pierde el efecto inhibitorio sobre la vasoconstricción dependiente de ECA y no se observa efecto antihipertensivo en ratas SHR tras su administración oral. Por el contrario, el efecto antihipertensivo tras la administración intravenosa pasa de ser transitorio (1-2 min, PACEI50L) a duradero (> 1 h, PACEI50D). Estos efectos en ratas también se observan con los estereoisómeros D y L de PACEI32, a pesar de no observarse diferencias significativas en potencia inhibitoria y efecto vasoactivo (Centeno y cols., 2006). También se han demostrado diferencias significativas en potencia inhibitoria dependiendo de la utilización de isómeros D- o L- en el caso de tri- y tetrapéptidos inhibidores de ECA derivados de caseína. Sin embargo, no se han abordado estudios *in vivo* que confirmaran la pérdida de efecto antihipertensivo en péptidos con configuración D (Maruyama y cols., 1987).

En su conjunto, estos resultados parecen indicar que si bien la configuración de los aminoácidos puede determinar la interacción con el centro activo de ECA, la secuencia aminoacídica y la posición de un

determinado aminoácido en la secuencia sigue siendo determinante. De hecho Oshima y Nagasawa, 1979, demostraron la estereoespecificidad de un residuo aminoácido en posición tres desde el extremo C-terminal pero no en posición cuatro.

La comparación entre los efectos antihipertensivos obtenidos en función del tipo de administración (oral o intravenosa) y la estereoselectividad también nos permite establecer ciertas conclusiones sobre la biodisponibilidad de los péptidos. El efecto antihipertensivo nulo de los péptidos PACEI32D y PACEI50D tras la administración oral frente a la reducción de presión arterial observada con PACEI32L y PACEI50L podría indicar que no hay absorción intestinal, o bien que el efecto antihipertensivo de los péptidos L se debe a posibles fragmentos derivados de la digestión gastrointestinal, que en el caso de los D-péptidos no se produce por su mayor resistencia a las proteasas. En el caso de la absorción intestinal, se ha descrito que dipéptidos con aminoácidos D presentan una menor absorción a través de los transportadores específicos de la mucosa intestinal que los péptidos L (Lister y cols., 1995), pero por el contrario, otros estudios han demostrado la absorción intestinal a través de la vía paracelular de D-octapéptidos intactos (Pappenheimer y cols., 1994). Los ensayos de administración intravenosa también confirman la mayor estabilidad de los D-péptidos frente a las peptidasas plasmáticas (Asano y Lübbehüsen, 2000), lo que explicaría el efecto antihipertensivo transitorio de los L-péptidos, perdido de forma casi instantánea debido a la degradación, frente al efecto antihipertensivo duradero provocado por los péptidos con D-aminoácidos. El efecto antihipertensivo transitorio observado en este trabajo tras la administración intravenosa de PACEI32L y PACEI50L también se ha descrito para péptidos derivados de germen de trigo (Matsui y cols., 2000).

## **8. Seguridad**

La ingesta de proteínas, así como de sus hidrolizados y péptidos individuales de dichos hidrolizados, o de péptidos diseñados *de novo*, plantea cuestiones de seguridad relacionadas con la toxicidad e ingesta excesiva, así como con la alergenicidad.

En relación a los péptidos derivados de proteínas alimentarias, en principio son considerados seguros ya que estas proteínas están presentes en la dieta. En particular, la lactoferrina bovina, está considerada alimento generalmente reconocido como seguro (GRAS) debido a su largo historial de uso en la dieta, así como por los estudios que verifican la seguridad de su administración en forma purificada (Wakabayashi y cols., 2006). Aunque en nuestro estudio no hemos analizado la toxicidad de su hidrolizado y de los péptidos individuales de dicho hidrolizado, existen en la bibliografía trabajos en los que se demuestra que los péptidos derivados de LfcinB no poseen actividad hemolítica (Nikawa y cols., 2004) y no ejercen un efecto tóxico frente a células animales (Wakabayashi y cols., 1999). Hidrolizados de caseína con reconocidos péptidos antihipertensivos se han demostrado también seguros. Diversos productos con lactotripéptidos (IPP y VPP) han pasado satisfactoriamente tests de toxicidad *in vitro* e *in vivo*, y no producen efectos adversos en ensayos clínicos con humanos (Boelsma y Kloeck., 2009). Por otra parte, el hidrolizado Lowpept® con los péptidos antihipertensivos RYLGY y AYFYPEL también ha superado ensayos de toxicidad aguda y en dosis mantenidas (Anadón y cols., 2010).

Otro punto importante a considerar es si una ingesta excesiva de proteínas lácteas, de sus hidrolizados o péptidos derivados podría tener consecuencias no deseables sobre la salud. Estudios epidemiológicos no confirman que una gran ingesta de leche por parte de individuos adultos se correlacione con enfermedades como hipercolesterolemia o enfermedades cardiovasculares (Usinger y cols., 2009), y por tanto no parece probable que los hidrolizados puedan tener relación con estas enfermedades, que en cualquier caso estarían relacionadas con el componente graso de la

leche más que con el componente proteico. La ingesta de 5 veces la dosis recomendada de tabletas de hidrolizado de caseína con lactotripéptidos no produce efectos colaterales adversos en pacientes hipertensos, ni presenta efecto rebote al suspender el tratamiento (Ishida y cols., 2011).

En cuanto a posibles reacciones alérgicas, una proporción elevada de la población debe prescindir de la leche en su dieta ya que presenta algún grado de intolerancia a alguna de sus proteínas. En este caso, una alternativa que se ha utilizado con éxito durante más de 50 años ha sido precisamente la utilización de hidrolizados de proteínas lácteas, los cuales se han mostrado seguros frente a alergias e intolerancias (Clemente, 2000). También es habitual la aplicación de hidrolizados de proteínas lácteas en nutrición deportiva (Schaafsma, 2009).

Mención aparte merecen los péptidos diseñados *de novo*, como los hexapéptidos y heptapéptidos PACEI, de los cuales no se puede presuponer la seguridad al no proceder de proteínas alimentarias. En nuestro estudio se han realizado ensayos de citotoxicidad con los PACEIs antihipertensivos PACEI32L y PACEI50L. Los resultados muestran que las potencias citotóxicas del hexa- y heptapéptido son mucho más bajas que sus potencias inhibitoras de la ECA. Por otra parte, las potencias citotóxicas de estos péptidos son también más bajas que la mostrada por la nisina, una bacteriocina de grado alimentario (Maher y McClean, 2006; Vaucher y cols., 2010).

## 9. Consideraciones finales

Este trabajo ha puesto de manifiesto el potencial de diferentes secuencias peptídicas, tanto derivadas de LF como de nuevo diseño, así como de hidrolizados de LF, como agentes antihipertensivos orales.

En estos momentos, la utilización de péptidos individuales, debido fundamentalmente a su coste, sería más factible en la industria farmacéutica que en la alimentaria. En este sentido, la producción biotecnológica de péptidos antihipertensivos utilizando metodologías de bajo coste y garantizando la seguridad biológica de los sistemas de producción sería clave. Hasta la fecha se han descrito varios sistemas de expresión, tanto procariotas (Chen y cols., 2008; Feng y cols., 2006; Guerreiro y cols., 2008; Park y cols., 2009; Xu y cols., 2007), como eucariotas (Chen y cols., 2009; Jin y cols., 2009; Yamada y cols., 1990; Zhang y cols., 2006) para la sobreexpresión de péptidos recombinantes. En el caso de péptidos antihipertensivos, la expresión en *E. coli* es la que más se ha explorado. En concreto, se ha descrito la expresión de un dodecapéptido derivado de caseína (FFVAPFPEVFGK) en forma de proteína de fusión (Lv y cols., 2003). Otra estrategia ha sido la expresión de péptidos antihipertensivos en forma de multímeros en tándem, como es el caso del tripéptido HHL derivado de soja (Jeong y cols., 2007) o el hexapéptido derivado de caseína KVLPVP (Liu y cols., 2007). Asimismo se ha descrito la producción de un péptido precursor de once péptidos antihipertensivos unidos en tándem y separados por puntos de corte de proteasas gastrointestinales (Rao y cols., 2009). Sin embargo, aunque estas estrategias son efectivas, el hecho de que los péptidos se produzcan fusionados a proteínas o que sean necesarios cortes con diferentes proteasas para su liberación dificulta y encarece su proceso de purificación. Diversos autores han especulado con la posibilidad de desarrollar cepas de bacterias lácticas capaces no solo de expresar péptidos antihipertensivos, sino también de entregarlos *in situ* en la mucosa intestinal. Sin embargo, los problemas de aceptación social

relativos a la utilización de organismos modificados genéticamente en agroalimentación, sobre todo en la Unión Europea, hacen que esta posibilidad tenga que ser considerada más como alternativa de futuro que de presente.

Este trabajo también plantea el empleo de hidrolizados enzimáticos de LF bovina como agente antihipertensivo oral. Nuestros resultados ponen de manifiesto que la fracción de peso molecular inferior a 3 KDa del hidrolizado de LF bovina obtenido con pepsina ejerce un efecto antihipertensivo en ratas SHR, lo que sugiere su potencial utilización como nutraceútico. En este contexto, sería más probable que tanto la industria como los consumidores consideraran la utilización de un hidrolizado de proteína láctea como alternativa más natural que la del empleo de péptidos individuales. En la actualidad, es posible obtener esta proteína a gran escala a partir de leche de bovino ó suero lácteo (Wakabayashi y cols., 2006), y también se ha descrito su producción en *A. oryzae* (Ward y cols., 1992) y *A. niger* var. *awamori* (Ward y cols., 1995), así como en leche de vacas modificadas genéticamente (van Berkel y cols., 2002). Sin embargo, esta propuesta tampoco está exenta de consideraciones adicionales. Además de los costes de obtención del hidrolizado, su incorporación a determinados alimentos podría dificultarse por las posibles repercusiones organolépticas, debido al potente sabor amargo que confieren los péptidos de carácter hidrofóbico y bajo peso molecular (Saha y Hayashi, 2001). También sería necesaria una evaluación de la resistencia de los péptidos a las diferentes condiciones de procesado de los alimentos. De hecho, se han descrito numerosos estudios donde se enfatiza la complejidad de las interacciones entre las matrices alimentarias y los péptidos bioactivos, que pueden afectar su estructura y bioactividad, e incluso modificar su biodisponibilidad (Hernández-Ledesma y cols., 2011).

Por último comentar que, sin lugar a dudas, los péptidos con efectos antihipertensivos han suscitado gran interés en la comunidad científica, en la industria alimentaria y entre los consumidores. Los efectos

## Discusión

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de los péptidos antihipertensivos se han evaluados en ensayos *in vitro*, en animales de experimentación y en humanos, y han sido incorporados a diferentes alimentos. En este contexto, los resultados controvertidos derivados de los ensayos clínicos y los requerimientos legales necesarios para aceptar las declaraciones nutricionales no pueden más que contribuir a que se siga investigando en esta área. En este sentido, se debe profundizar en aquellos estudios que demuestren la estabilidad de los péptidos, su absorción y cual es forma activa en el organismo. Al igual que en el caso de los compuestos farmacéuticos, será imprescindible la determinación de la concentración relevante a nivel fisiológico así como la cinética de eliminación de los péptidos antihipertensivos. Estos estudios se verán favorecidos por la aplicación de nuevas técnicas analíticas así como del avance de nuevas disciplinas como la nutrigenómica y la nutrigenética que permitirán la identificación de nuevos biomarcadores de exposición y actividad.



## **CONCLUSIONES**

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1. Se ha demostrado el potencial de péptidos derivados de distintas zonas de la secuencia de la lactoferrina bovina (LFb), incluido su dominio antimicrobiano lactoferricina (LfcinB), para controlar la hipertensión.
2. El hidrolizado de LFb con pepsina muestra efecto antihipertensivo en ratas espontáneamente hipertensas (SHRs) tras administración oral sólo si es ultrafiltrado para obtener la fracción de péptidos de tamaño molecular inferior a 3 KDa (LFH < 3 KDa), lo cual indica que el efecto antihipertensivo debe atribuirse principalmente al componente peptídico de bajo peso molecular.
3. La hidrólisis de LFb con pepsina genera péptidos antihipertensivos de secuencia RPYL, LIWKL y LNNSRAP que no habían sido descritos anteriormente. Entre ellos destaca LIWKL por mantener su efecto antihipertensivo en SHRs durante 24 h, a pesar de administrarse oralmente de manera aguda en dosis única.
4. Dos péptidos derivados del dominio LfcinB, el hexapéptido LfcinB<sub>20-25</sub> y su fragmento WQ, tienen efecto antihipertensivo en SHRs, aunque no se ha podido determinar con certeza quién es el responsable de la disminución de la presión arterial tras la administración oral del hexapéptido.
5. LfcinB<sub>20-25</sub>, como otras secuencias derivadas de proteínas lácteas, puede considerarse un péptido multifuncional que ejerce efectos antihipertensivos y antimicrobianos.
6. Se han obtenido heptapéptidos mediante diseño racional que mejoran las propiedades inhibitoras sobre la enzima convertidora de angiotensina I (ECA) cuando se comparan con los hexapéptidos parentales.

## Conclusiones

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7. Uno de los hexapéptidos parentales, PACEI32L, y dos heptapéptidos, PACEI50L y PACEI52L, muestran efectos antihipertensivos en SHRs tras su administración oral, a pesar de que son susceptibles de degradación por las proteasas plasmáticas. Los efectos son similares a los producidos por el fármaco de referencia captopril. Ninguno de los tres péptidos muestra efecto hipotensor tras su administración oral a ratas normotensas, ni, en el caso de PACEI32L y PACEI50L, efectos citotóxicos.

8. Las aproximaciones experimentales utilizadas en este trabajo ponen de manifiesto la falta de correlación entre los resultados obtenidos en los ensayos *in vitro* de inhibición enzimática, *ex vivo* de vasoactividad en arterias aisladas e *in vivo* con un modelo animal de hipertensión, lo que indica la compleja relación existente entre la secuencia peptídica y su actividad biológica, y por tanto la necesidad de realizar los tres tipos de ensayos para analizar el potencial antihipertensivo de los péptidos bioactivos.

9. La inhibición de la actividad ECA es el mecanismo de acción responsable al menos en parte de los efectos antihipertensivos del péptido derivado de lactoferricina LfcinB<sub>20-25</sub>, del hexapéptido PACEI32L, de los heptapéptidos PACEI50L y PACEI52L, así como del hidrolizado de lactoferrina LFH < 3 KDa y de sus péptidos RPYL y LIWKL. Por el contrario, el efecto antihipertensivo mostrado por el dipéptido WQ, fragmento de degradación gastrointestinal de LfcinB<sub>20-25</sub>, y del péptido LNNSRAP derivado de LF no puede explicarse a través de un efecto inhibitor de la actividad ECA.

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## **ANEXOS**

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## LISTA DE PUBLICACIONES

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Esta tesis es una compilación de los cuatro artículos originales publicados que se indican a continuación:

- I. Antihypertensive properties of lactoferricin B-derived peptides. **Ruiz-Giménez, P.**, Ibañez, A., Salom, J.B., Marcos, J.F., López-Díez, J.J., Vallés, S., Torregrosa, G., Alborch, E. and Manzanares, P. *Journal of Agricultural and Food Chemistry*, 58, 6721-6727, 2010.
- II. Bovine lactoferrin pepsin hydrolysate exerts inhibitory effect on angiotensin I-converting enzyme-dependent vasoconstriction. **Ruiz-Giménez, P.**, Burguete, M.C., Castelló-Ruiz, M., Marcos, J.F., Salom, J.B., Vallés, S., Torregrosa, G., Alborch, E. and Manzanares, P. *International Dairy Journal*, 17, 1212-1215, 2007.
- III. Antihypertensive effect of a bovine lactoferrin pepsin hydrolysate: Identification of a novel active peptides. **Ruiz-Giménez, P.**, Salom, J.B., Marcos, J.F., Vallés, S., Martínez-Maqueda, D., Recio, I., Torregrosa, G., Alborch, E. and Manzanares, P. *Food Chemistry*, 131, 266-273, 2012.
- IV. Novel antihypertensive hexa- and heptapeptides with ACE-inhibiting properties: From the in vitro ACE assay to the spontaneously hypertensive rat. **Ruiz-Giménez, P.**, Marcos, J.F., Torregrosa, G., Lahoz, A., Fernández-Musoles, R., Vallés, S., Alborch, E., Manzanares, P. and Salom, J.B. *Peptides*, 32, 1431-1438, 2011.



**ANEXO I**

**Antihypertensive properties of lactoferricin B-  
derived peptides**

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López-Díez, J.J., Vallés, S., Torregrosa, G., Alborch, E.  
and Manzanares, P

*Journal of Agricultural and Food Chemistry*, 58, 6721-6727, 2010





## Antihypertensive Properties of Lactoferricin B-Derived Peptides

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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<sub>20–25</sub>, 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<sub>50</sub> 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<sub>20–25</sub> 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<sub>20–25</sub>. 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).

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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,



**ANEXO II**

**Bovine lactoferrin pepsin hydrolysate exerts  
inhibitory effect on angiotensin I-converting  
enzyme-dependent vasoconstriction**

Ruiz-Giménez, P., Burguete, M.C., Castelló-Ruiz, M., Marcos,  
J.F., Salom, J.B., Vallés, S., Torregrosa, G., Alborch, E.  
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*International Dairy Journal*, 17, 1212-1215, 2007.





## Bovine lactoferrin pepsin hydrolysate exerts inhibitory effect on angiotensin I-converting enzyme-dependent vasoconstriction

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### Abstract

The inhibitory effect of a pepsin hydrolysate of bovine lactoferrin (LFH) on angiotensin I-converting enzyme (ACE) has been examined using in vitro and ex vivo functional assays. In vitro assays showed a LFH inhibitory effect on ACE activity with an  $IC_{50}$  value of  $0.95 \pm 0.06$  mg mL<sup>-1</sup>. Ex vivo functional assays using rabbit carotid artery segments showed a LFH inhibitory effect on ACE-dependent angiotensin I-induced contraction, but not on angiotensin II-induced contraction, suggesting that the effect of LFH is not due to antagonism of receptors for angiotensin II. LFH was shown to possess ACE inhibitory effect with potential to modulate hypertension, although the possible inhibitory effect of LFH on angiotensinases deserves further research.

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**Keywords:** Bovine lactoferrin; Pepsin hydrolysate; ACE inhibition; ACE-dependent vasoconstriction; Hypertension

### 1. Introduction

Lactoferrin (LF), a major constituent of mammalian milk, is a multifunctional iron glycoprotein which is known to exhibit a diverse range of biological effects, including antimicrobial, antiviral, antioxidant, and immunomodulatory activities (Tomita, Wakabayashi, Yamauchi, Teraguchi, & Hayasawa, 2002). It is now accepted that enzymatic hydrolysis of food protein releases peptides that may exhibit different or enhanced biological activities. In this context, pepsin-digested LF has been found more effective than LF as an antimicrobial against several bacteria, showing a bactericidal effect instead of the bacteriostatic effect of LF (Tomita et al., 1991). The active peptides responsible for the antimicrobial activity of the LF

hydrolysate were subsequently identified (Bellamy et al., 1992) and were named lactoferricin H from human milk and lactoferricin B from bovine milk (LfcinB). Moreover, two additional antibacterial peptides different from Lfcin have been isolated from a pepsin digest of bovine LF (Dionysius & Milne, 1997).

The renin-angiotensin system is a coordinated peptidic hormonal cascade for the control of cardiovascular, renal, and adrenal functions governing fluid and electrolyte balance, and arterial blood pressure (Carey & Siragy, 2003). Briefly, prorenin is converted to active renin by a trypsin-like enzyme. Renin cleaves angiotensinogen to form angiotensin I. Angiotensin I-converting enzyme (ACE) hydrolyses both the inactive angiotensin I into vasoconstrictor angiotensin II and the vasodilator bradykinin into an inactive metabolite. Most well-known effects of angiotensin II, including vasoconstriction, are mediated by angiotensin type 1 receptors (AT<sub>1</sub>). Angiotensin II also

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**ANEXO III**

**Antihypertensive effect of a bovine lactoferrin  
pepsin hydrolysate: Identification of a novel  
active peptides**

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Maqueda, D., Recio, I., Torregrosa, G., Alborch, E. and  
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## Antihypertensive effect of a bovine lactoferrin pepsin hydrolysate: Identification of novel active peptides

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### ABSTRACT

The potential of bovine lactoferrin (LF) as a source of antihypertensive peptides has been examined. For this purpose, LF pepsin hydrolysate with molecular mass lower than 3 kDa (LFH < 3 kDa) was prepared and orally administered to spontaneously hypertensive rats (SHR), resulting in reduced systolic blood pressure in a significant and maintained manner up to 24 h after administration. LFH < 3 kDa was further fractionated by semi-preparative high performance liquid chromatography (HPLC) and 38 peptides, contained in the active fractions, were identified by using an ion trap mass spectrometer. Based on the peptide abundance, a total of 11 peptides were chemically synthesized and their ACE inhibitory activity tested. Only three of them, corresponding to peptides of sequences LIWKL, RPVL and LNNSRAP exerted *in vitro* inhibitory effects on angiotensin I converting enzyme (ACE) activity and had a 50% inhibitory concentration (IC<sub>50</sub>) of 0.47, 56.5 and 105.3 μM, respectively. The three peptides also showed antihypertensive effects in SHR and remarkably the effect of LIWKL remained significant for up to 24 h post-administration, similarly LFH < 3 kDa and the captopril control. The two most potent *in vitro* inhibitory peptides showed *ex vivo* inhibitory effect on ACE-dependent vasoconstriction as well. In conclusion, three novel LF-derived peptides and a pepsin LFH < 3 kDa lowered blood pressure and exhibit potential as orally effective antihypertensive compounds.

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### 1. Introduction

Over the past two decades increasing scientific and industrial interest has been focused on the biological properties of milk proteins, which possess additional physiological effects due to the numerous bioactive peptides that are encrypted within intact proteins. Those peptides, once released, exhibit different activities affecting the digestive, cardiovascular, immune and nervous systems (Korhonen & Pihlanto, 2006). Among them, those with blood pressure-lowering effects are receiving increasing attention due to the prevalence and importance of hypertension in the Western population (Ricci, Artacho, & Olalla, 2010). The antihypertensive effect of milk protein derived peptides seems to be mainly due 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 bradykinin into an inactive peptide (Campbell, 2003) leading to blood pressure upregulation.

In this context, technological processes for the isolation and enrichment of bioactive peptides have been developed and proven effective. Basically, processing of milk proteins with: (i) food grade proteolytic preparations of plant, fungal or microbial origin or digestive enzymes or (ii) fermentation of milk with proteolytic starters have been employed to release ACE inhibitory peptides. Both approaches have conducted due to the development of commercial products based on casein or whey proteins with antihypertensive effects in humans (De Leo, Panarese, Gallerani, & Ceci, 2009). In fact, the most popular functional foods contain the casein-derived ACE-inhibitory tripeptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), obtained by means of either milk fermentation

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**ANEXO IV**

**Novel antihypertensive hexa- and heptapeptides  
with ACE-inhibiting properties: From the *in vitro*  
ACE assay to the spontaneously hypertensive rat**

Ruiz-Giménez, P., Marcos, JF., Torregrosa, G., Lahoz, A.,  
Fernández-Musoles, R., Valles, S., Alborch, E., Manzanares,  
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*Peptides*, 32, 1431-1438, 2011





## Novel antihypertensive hexa- and heptapeptides with ACE-inhibiting properties: From the in vitro ACE assay to the spontaneously hypertensive rat

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### ABSTRACT

Bioactive ACE inhibiting peptides are gaining interest in hypertension treatment. We have designed and screened six synthetic heptapeptides (PACEI48 to PACEI53) based on two hexapeptide leads (PACEI32 and PACEI34) to improve ACE inhibitory properties and assess their antihypertensive effects. ACE activity was assayed in vitro and ex vivo. Selected peptides were administered to spontaneously hypertensive rats (SHRs) and normotensive Wistar-Kyoto (WKY) rats. In vitro cytotoxicity was assessed with the MTT reduction test. The six heptapeptides at low micromolar concentration produced different degrees of in vitro inhibition of ACE activity using the synthetic substrate HHL or the natural substrate angiotensin I; and ex vivo inhibition of ACE-dependent, angiotensin I-induced vasoconstriction, but not angiotensin II-induced vasoconstriction. Oral administration of the hexapeptide PACEI32L and the heptapeptides PACEI50L and PACEI52L, induced reductions in systolic blood pressure lasting up to 3 h in SHRs but not in WKY rats. Intravenous injection of PACEI32L and PACEI50L, but not PACEI52L, induced acute transient reductions in mean blood pressure of SHRs. D-Amino acid peptides showed five-fold less ACE inhibitory potency, no inhibitory effect on angiotensin I-induced vasoconstriction, and antihypertensive effect in SHRs after i.v. injection, but not after oral administration. The toxicity of peptides to reduce the viability of cultured cells was in the millimolar range. In conclusion, we have obtained novel rationally designed heptapeptides with improved ACE inhibitory properties when compared to lead hexapeptides. One selected hexapeptide and two heptapeptides show oral antihypertensive effects in SHRs and appear safe in cytotoxicity assays.

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### 1. Introduction

The increase in cardiovascular risk inherent to hypertension leads to premature morbidity and mortality [10]. Prolonged blood pressure lowering through lifestyle interventions or pharmacological treatments is associated with reductions in the risks of stroke (30–40%) and coronary events (20%), and appears to reduce to a lesser degree the incidences of other complications [31]. Since

dysfunctions in the renin–angiotensin–aldosterone system (RAAS) are involved in the pathophysiology of hypertension [3], several antihypertensive drugs target on this system at different points of the cascade: renin inhibitors, angiotensin converting enzyme (ACE) inhibitors, AT1 receptor blockers and aldosterone receptor antagonists [31]. ACE inhibition is also the goal of antihypertensive peptides derived from plant and animal proteins such as milk, soy or fish. These peptides can be enzymatically released from precursor proteins during food processing and gastrointestinal digestion. They may be generated in or incorporated into functional foods for the development of 'natural' beneficial health products, some of which are currently on the market or in development [7]. Lactotripeptides (IPP and VPP), the best characterized ACE inhibitor

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