INSTITUTO DE AGROQUÍMICA Y TECNOLOGÍA DE ALIMENTOS

INSTITUTO DE INVESTIGACIÓN SANITARIA LA FE

UNIVERSITAT DE VALÈNCIA

Departament de Medicina Preventiva i Salut Pública, Ciències de l'Alimentació, Toxicologia i Medicina Legal

Programa de Doctorado en Ciencias de la Alimentación

PÉPTIDOS ANTIHIPERTENSIVOS DERIVADOS DE PROTEÍNAS LÁCTEAS: PRODUCCIÓN MEDIANTE LEVADURAS NO CONVENCIONALES Y MECANISMOS DE ACCIÓN

Memoria presentada por: **Aurora García Tejedor**

Directores de Tesis:

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PARA OPTAR AL GRADO DE DOCTOR POR LA UNIVERSITAT DE VALÈNCIA

Valencia, septiembre 2015

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CERTIFICAN: Que Dña. Aurora García Tejedor, Licenciada en Ciencia y Tecnología de los Alimentos por la Universidad de Valencia, ha realizado bajo su codirección el trabajo titulado: "Péptidos antihipertensivos derivados de proteínas lácteas: producción mediante levaduras no convencionales y mecanismos de acción", que presenta para optar al grado de Doctor en el programa de Ciencias de la Alimentación por la Universitat de València.

Y para que así conste a los efectos oportunos, firman el presente certificado en Valencia, a 17 de septiembre de 2015.

Dra. Paloma Mª Manzanares Mir

Dr. Juan B. Salom Sanvalero

El trabajo que aquí se expone se ha llevado a cabo en el Departamento de Biotecnología de los Alimentos del IATA (CSIC) y en el Centro de Investigación del Hospital Universitario y Politécnico “La Fe”. Este trabajo ha sido posible gracias a la concesión de una ayuda predoctoral de formación de personal investigador, Pre-Doc FPI (BES-2011-044424) asociada al proyecto AGL2010-21009 del Ministerio de Ciencia e Innovación. Esta Tesis Doctoral se enmarca también dentro del proyecto Consolider Ingenio Fun-C-Food CSD2007-00063. A su vez, he de agradecer la concesión de dos ayudas a la movilidad que me permitieron realizar sendas estancias breves, una en el Instituto de Investigación en Ciencias de la Alimentación (CIAL CSIC-UAM, Madrid) y otra en el Institute of Food Research (IFR, Norwich, Reino Unido), concedidas por el Ministerio de Ciencia e Innovación y el Ministerio de Economía y Competitividad respectivamente.

Agradecimientos

Ésta es una de las partes más complicadas de escribir en una tesis. Y es que: ¿Cómo agradecer a todas las personas que han pasado por tu vida y te han aportado tanto durante estos años? ¿Cómo comprimirlo en unos sencillos folios? Tarea complicada, pero a la vez gratificante.

En primer lugar, me gustaría agradecer de manera muy especial a mis directores de tesis, la Dra. Paloma Manzanares y el Dr. Juan B. Salom. Gracias por brindarme la posibilidad de realizar esta tesis doctoral y gracias por toda la confianza depositada en mí desde el primer día. Pero, sobre todo, gracias por enseñarme tanto y por estar a mi lado en todo momento, tanto profesional como personalmente. No podría haber tenido mejores directores de tesis.

También me gustaría dar las gracias a dos personas que, sin ser mis directores de tesis, se han comportado como tal en diferentes ocasiones. Dr. José Gimeno, gracias por estar siempre dispuesto a ayudarme. Me has enseñado mucho. Dr. Salvador Vallés, me hubiera gustado compartir contigo toda esta experiencia, ya que aunque fue poco tiempo, fue suficiente como para tener un gran recuerdo tuyo. Gracias por todos los consejos que me diste.

Esta tesis doctoral se ha llevado a cabo en dos centros diferentes: el Instituto de Agroquímica y Tecnología de Alimentos (IATA) y el Hospital Universitario y Politécnico “La Fe”; además de tres estancias, lo que me ha permitido conocer a una gran cantidad de gente que me ha aportado mucho.

En primer lugar, el IATA, donde he pasado la mayor parte del tiempo y donde he tenido el placer de conocer a grandes personas y sobre todo, grandes amigos. Mis compañeros de laboratorio Bea, Javier, Meri, Ricardo y, aunque por menos tiempo y no por ello menos importantes, Marlen y Josep: gracias por todos los momentos compartidos, por ese

buen ambiente que creábais y por conseguir que ir al laboratorio siempre fuese algo divertido.

Isaac, el IATA nos unió y sé que serás un amigo para toda la vida. Aunque no esperes que te dé las gracias: gracias por todo. Por todas las risas, en el laboratorio y fuera de él y, sobre todo, gracias por escucharme siempre.

A mis amigos Anto, Ceci, Javi, Fani, Jiri, Alba, Adri y Sara. No separo vuestros agradecimientos porque para mí sois un todo y sin cada uno de vosotros esta experiencia no habría sido igual. Gracias por estar ahí y por hacer que fuese tan fácil sonreír. Gracias por convertir cada pequeño acontecimiento en una gran fiesta. Habéis sido como una gran familia y lo seguiréis siendo.

María, Ana, Esther, Amparito, Clara, Lidia y Lorena gracias por ser tan buenas compañeras y mejores personas. No me quiero olvidar de toda la gente del IATA que me ha encantado conocer y con los que he disfrutado de tantos momentos: Vicky, Ali, Eli, David, Jesús, Vicente, Rosa, Migue, Dulce, Walter, Patri, Carmela, Sergi, José Manuel, Andrew, Estefanía, Luisa, Amparo y un largo etcétera, gracias por todo lo que me habéis aportado. Así como a todos los integrantes del departamento de Proteómica y Genómica del IATA y del departamento de Fisiología de la Facultad de Farmacia. Finalmente, gracias al Dr. José Vicente Gil, ya que fue él quien despertó en mí el interés por la investigación.

Por otro lado, está mi gente de “La Fe”: Chelo, María, Micaela y Sonia, ¿qué puedo decir de vosotras? Hemos compartido mucho, tanto risas como llantos. Gracias por hacer que siempre tuviera ganas de una época más en “La Fe”. Simplemente, gracias. También me gustaría dar las gracias al Dr. Germán Torregrosa y al Dr. Enrique Alborch, por el buen ambiente de trabajo que han creado siempre.

Me gustaría agradecer la posibilidad que me brindó la Dra. Isidra Recio por permitirme realizar una estancia en el Instituto de Investigación

en Ciencias de Alimentación, así como por toda la atención mostrada durante esos meses y siempre que la he necesitado. Gracias a todas las personas que conocí durante esta estancia, especialmente a Alberto, Laura, Elvia, Dani, Samuel, Mónica, Bea, Almudena, Sara, Laura, ya que me acogisteis con los brazos abiertos e hicisteis que Madrid se convirtiera en mi segunda casa. También me gustaría agradecer a la Dra. Dolores Prieto y a todo su equipo de la Universidad Complutense de Madrid porque en muy poco tiempo me enseñaron mucho. Así como al Dr. Paul Kroon y a todos los miembros de su grupo del Institute of Food Research por permitirme aprender tanto en mi última estancia.

También me gustaría agradecer a dos personas que me han ayudado mucho. A mi prima Elvira, por ayudarme con el formato de esta tesis a una velocidad indescriptible y, sobre todo, por estar siempre ahí. Y a mi amigo Paco, por el gran diseño de la portada y por todo lo que me aporta siempre. También me gustaría dar las gracias a mis amigos de siempre y a mi familia, esas personas que nunca me han fallado y con las que siempre podré contar. Y a mis amigos del Erasmus, que después de siete años siguen ahí y ya sé que se van a quedar para siempre.

Finalmente, el mayor de mis agradecimientos es para mis padres, por todo lo que me han enseñado, por su apoyo incondicional en todo lo que hago y por darme tantas oportunidades en la vida. Gracias por convertirme en la persona que soy. También quiero dar las gracias a mi hermano y a Sandra, me animan en todo lo que hago y están siempre a mi lado cuando los necesito. Por último, quiero dar las gracias a Paco: aunque apareciste en mi vida no hace demasiado tiempo, te has convertido en uno de mis principales apoyos. Gracias por estar a mi lado tanto en los buenos como en los malos momentos.

En resumen, gracias a todos los que formáis parte de mi vida porque sin vosotros en ella, sin lo que me aportáis, cada una de las palabras que he escrito no existiría. Esta tesis es también vuestra.

¿Qué sería la vida si no

tuviéramos el valor de

intentar cosas nuevas?

Vincent van Gogh

RESUMEN

En esta Tesis Doctoral se ha estudiado el potencial de levaduras no convencionales, pertenecientes a las especies *Debaryomyces hansenii*, *Kluyveromyces lactis* y *Kluyveromyces marxianus,* para generar hidrolizados y péptidos antihipertensivos provenientes de caseína (CN) o lactoferrina (LF). Además, se ha profundizado en el mecanismo de acción responsable del efecto antihipertensivo. Para ello, se han utilizado diferentes aproximaciones experimentales que incluyen ensayos *in vitro* para determinar los efectos inhibitorios sobre la actividad de la enzima conversora de angiotensina (ECA) y ensayos *in vivo* en ratas espóntaneamente hipertensas (SHRs) para estudiar los efectos sobre la presión arterial (PA) tras administración oral. Además, se ha utilizado un modelo de ratas normotensas a las que se ha inducido la hipertensión mediante la administración de angiotensina I (Ang I) y angiotensina II (Ang II). En algunos casos, se han determinado los niveles circulantes de ECA, de Ang II y de aldosterona en sueros de SHRs. También se han empleado células endoteliales humanas para estudiar el efecto de los péptidos sobre la expresión génica.

Se generaron hidrolizados de CN y LF utilizando distintas cepas de levaduras. Estos hidrolizados mostraron diferente capacidad de inhibición de la ECA *in vitro*, y los más potentes se evaluaron *in vivo* en SHRs, provocando reducciones significativas de la presión arterial sistólica (PAS). En el caso de los hidrolizados de CN, se evaluó si las levaduras eran capaces de producir secuencias peptídicas concretas (IPP, VPP, RYLGY, AYFYPEL, LHLPLP y HLPLP), cuyo potencial antihipertensivo estaba ampliamente contrastado. Sólo se detectaron las secuencias LHLPLP y HLPLP, que fueron generadas por las cepas de *D. hansenii* Dh1 y Dh14. En el caso de la LF, se seleccionó el hidrolizado antihipertensivo más

potente, concretamente el producido con *K. marxianus* Km2; se identificaron los péptidos más abundantes, y se seleccionaron las secuencias DPYKLRP, PYKLRP, YKLRP y GILRP que mostraron potencia inhibitoria *in vitro* de la ECA y efecto antihipertensivo en SHRs. Además, con el objetivo de establecer relaciones secuencia-función, se incluyeron en la caracterización los péptidos KLRP y LRP que también mostraron dichos efectos. En concreto, las dos secuencias con mayor efecto antihipertensivo, DPYKLRP y LRP redujeron, en suero de SHRs, la actividad ECA circulante, así como los niveles de Ang II y aldosterona. La inhibición *in vivo* de la ECA de determinados péptidos derivados de LF fue confirmada utilizando modelos de hipertensión inducida con Ang I. Además, estos experimentos indicaron un papel menos relevante de la inhibición de los receptores de Ang II en el efecto antihipertensivo. Finalmente, en el modelo celular HUVEC, se observó que un hidrolizado enzimático de LF fue capaz de modificar la expresión de genes de la vía de producción y señalización de óxido nítrico (NO), concretamente los genes NOSTRIN y GUCY, y de aumentar la producción de NO. Estos ensayos también indicaron la posible contribución de las prostaglandinas.

En conclusión, se ha demostrado la posibilidad de utilizar levaduras GRAS (Generally Recognized As Safe) para la producción de hidrolizados y péptidos con efecto antihipertensivo provenientes de las proteínas lácteas CN y LF. Los hidrolizados y péptidos derivados de LF actuarían principalmente a través del sistema renina-angiotensina-aldosterona (SRAA), mediante la inhibición de la ECA y en menor medida a través del bloqueo de los receptores de Ang II, poniéndose también de manifiesto la relevancia del sistema NO.

ÍNDICE

ABREVIATURAS

Ang I	Angiotensina I
Ang II	Angiotensina II
ASS	Argininosuccinato sintetasa
AT_1	Receptor de angiotensina tipo 1
AT_2	Receptor de angiotensina tipo 2
BAL	Bacterias ácido lácticas
BSA	Bovine Serum Albumin (seroalbúmina bovina)
B_1	Receptor de bradiquinina tipo 1
B_2	Receptor de bradiquinina tipo 2
CN	Caseína
COX-1	Ciclooxigenasa 1
COX-2	Ciclooxigenasa 2
C-terminal	Carboxilo terminal
ECA	Enzima conversora de angiotensina
ECE	Enzima conversora de endotelina
EFSA	European Food Safety Authority (Autoridad Europea de Seguridad Alimentaria)
eNOS	Endothelial Nitric Oxide Synthase (óxido nítrico sintasa isoforma endotelial)
ET	Endotelina
ET_A	Receptor de endotelina-1 tipo A

ET_B	Receptor de endotelina-1 tipo B
GCs	Guanilato ciclasa soluble
GMPc	Guanosín monofosfato cíclico
GRAS	Generally Recognized As Safe (generalmente reconocida como segura)
GUCY1A3	Guanilato ciclasa 1 subunidad α3
HTA	Hipertensión arterial
HUVEC	Human Umbilical Vein Endothelial Cells (células endoteliales de la vena umbilical humana)
Ig	Inmunoglobulinas
iNOS	Inducible Nitric Oxide Synthase (óxido nítrico sintasa isoforma inducible)
α-La	α-Lactoalbúmina
β-Lg	β-Lactoglobulina
LF	Lactoferrina bovina
Lfcin	Lactoferricina
LFH	Lactoferrin hydrolysate (hidrolizado de lactoferrina)
nNOS	Neuronal Nitric Oxide Synthase (óxido nítrico sintasa isoforma neuronal)
NO	Nitric Oxide (óxido nítrico)
NOS	Nitric Oxide Synthase (óxido nítrico sintasa)
NOSTRIN	Nitric Oxide Synthase Trafficking (traslocador de la óxido nítrico sintasa)

N-terminal	Amino terminal
PA	Presión arterial
PAD	Presión arterial diastólica
PAS	Presión arterial sistólica
PCR	Polymerase Chain Reaction (reacción en cadena de la polimerasa)
PTGS2	Prostaglandina-endoperóxido sintasa 2
Q-TOF	Quadrupole Time-Of-Flight mass spectrometer (espectrometría de masas de alta resolución)
SHRs	Spontaneously Hypertensive Rats (ratas espontáneamente hipertensas)
SRAA	Sistema Renina-Angiotensina-Aldosterona
UPLC	Ultra Performance Liquid Chromatography (cromatografía líquida de ultraresolución)
WKY	Rata Wistar-Kyoto

Los aminoácidos se identifican con el código de tres letras cuando aparecen de forma individual y con el código de una letra en las secuencias peptídicas.

Introducción

Los alimentos funcionales, aquellos que además de su valor nutricional, poseen un efecto beneficioso sobre una o varias funciones específicas en el organismo (Functional Food Science in Europe, FUFOSE, 1999), han tenido una gran aceptación en el mercado debido a la preocupación que tiene el consumidor por el binomio alimentación y salud.

Debido al incremento constante de este tipo de productos en el mercado, fue necesario un marco regulador que protegiera a los consumidores de las alegaciones falsas o confusas, y respondiera a las necesidades de la industria en cuanto a innovación, comercialización y promoción de nuevos productos. Por ello, la Unión Europea adoptó el Reglamento Nº 1924/2006 del Parlamento Europeo y del Consejo, de 20 de diciembre de 2006, relativo a las declaraciones nutricionales y de propiedades saludables en los alimentos (European Food Information Council, EUFIC, 2014), siendo la Autoridad Europea de Seguridad Alimentaria (EFSA) el agente gubernamental que revisa estas alegaciones. Según este reglamento, para que un alimento cumpla con los requisitos en materia de alimentos funcionales es necesario que tenga identificadas y cuantificadas las sustancias activas. También es necesario que se hayan realizado extensas investigaciones para probar el efecto funcional tanto en animales como en humanos, indicando la dosis mínima a la que se observa efecto y demostrando el mecanismo de acción.

El empleo de péptidos bioactivos como ingredientes funcionales antihipertensivos ha despertado un enorme interés debido a la relación de la hipertensión con las enfermedades cardiovasculares y la elevada incidencia de éstas en la población occidental. En este contexto se enmarcan los objetivos de la presente tesis sobre la generación de hidrolizados y secuencias peptídicas antihipertensivas derivados de caseína y lactoferrina bovina, empleando levaduras no convencionales, microorganismos poco explorados hasta la fecha para este uso. Además

se ha profundizado en el estudio de su mecanismo de acción, que no se limita a la inhibición de la enzima conversora de angiotensina.

1. Hipertensión arterial

1.1 Definición y relevancia clínica

La hipertensión arterial (HTA) se describe como una elevación crónica de la presión arterial (PA). Se considera que un individuo es hipertenso cuando la presión arterial sistólica (PAS) es igual o superior a 140 mm Hg y/o la presión arterial diastólica (PAD) es igual o superior a 90 mm Hg (WHO, *WHO/DCO/WHD/2013.2*, 2013).

La HTA es un trastorno muy serio, ya que incide directamente en la salud, considerándose la señal de alerta de un mayor riesgo cardiovascular: problemas cardíacos (infarto, angina o insuficiencia cardiaca), renales (insuficiencia renal) y cerebrales (hemorragia o infarto cerebral y, a la larga, demencia). La enfermedad cardiovascular causa a nivel mundial aproximadamente 17 millones de muertes al año, casi un tercio del total. La HTA es responsable de al menos el 45% de las muertes debidas a enfermedades del corazón, y el 51% de las muertes por accidente cerebrovascular (WHO, *WHO/DCO/WHD/2013.2*, 2013). Además, se considera que la prevalencia de HTA está en torno a un 30-45%, con un marcado aumento a edades más avanzadas (Mancia et al., 2013). Por tanto, la HTA es un problema grave de salud, sobre todo en los países desarrollados, suponiendo un elevado coste tanto a la sociedad como a los gobiernos.

Los tratamientos farmacológicos, administrados individualmente o combinados, son eficaces en la reducción de la PA, así como de la morbilidad y la mortalidad por enfermedades cardiovasculares y renales.

Los fármacos que más comúnmente se emplean son diuréticos, bloqueadores beta, antagonistas de los canales de calcio e inhibidores del sistema renina-angiotensina-aldosterona (SRAA). Sin embargo, el tratamiento farmacológico a largo plazo puede conllevar efectos adversos y requiere supervisión médica continua (Mancia et al., 2013).

Por otro lado, el estilo de vida es clave para el desarrollo de la HTA. El consumo elevado de sal y alcohol, el sobrepeso, el sedentarismo y el tabaco son algunos de los factores que influyen directamente en la aparición de la HTA. Se ha demostrado que una dieta rica en frutas, vegetales y productos lácteos bajos en calorías, así como un incremento de la actividad física, reducen la PA en pacientes hipertensos (Jauhiainen y Korpela, 2007). Por tanto, los cambios en el estilo de vida son fundamentales para el tratamiento de la HTA, aunque el mantenimiento de estos hábitos saludables a largo plazo es difícil.

Los tratamientos farmacológicos o cambios en el estilo de vida que provocan una bajada de los valores de PA en la HTA tienen un considerable impacto sobre el riesgo de sufrir enfermedades cardiovasculares (Boelsma y Kloek, 2009). Se ha estimado que una reducción de la PAS de tan solo 2 mm Hg se traduce en una reducción del 6% en accidentes cerebrovasculares y un 4% en enfermedades coronarias (Whelton et al., 2002).

1.2 Mecanismos de control de la presión arterial (PA)

La PA tiene un sistema de regulación complejo a través de diferentes mecanismos interrelacionados a corto, medio y largo plazo. A corto plazo, el sistema nervioso autónomo corrige desviaciones agudas de la PA controlando el gasto cardiaco y la resistencia periférica. A medio y

largo plazo, ejercen su función múltiples controles nerviosos y hormonales, como el sistema de control renal y de los líquidos corporales, así como diferentes mecanismos entre los que destacan el SRAA, el sistema quinina-calicreína, el sistema óxido nítrico (NO)-endotelina (ET) y el sistema de los péptidos natriuréticos (FitzGerald et al., 2004; Guyton y Hall., 2011).

1.2.1 Sistema renina-angiotensina-aldosterona (SRAA)

El SRAA es uno de los sistemas más importantes que intervienen en la regulación del tono vascular, el balance hídrico-electrolítico y la PA, y es una de las principales dianas para el tratamiento de la HTA.

El funcionamiento del SRAA se esquematiza en la Figura 1: cuando la PA disminuye, se libera renina que en el riñón convierte el angiotensinógeno, péptido de 14 aminoácidos, en angiotensina I (Ang I), un decapéptido de secuencia DNVYIHPFHL sin actividad biológica. En este punto, la enzima conversora de angiotensina (ECA) convierte la Ang I en angiotensina II (Ang II), un potente octapéptido vasoconstrictor (DNVYIHPF) y regulador del equilibrio de líquidos y de sodio (Oparil y Haber, 1974; Majumder y Wu, 2014). La ECA es la enzima clave en este sistema de regulación. Es una dipeptidil carboxipeptidasa dependiente de Zn^{2+} y Cl^- que cataliza la hidrólisis de dipéptidos del extremo carboxilo terminal (C-terminal) de una gran variedad de péptidos, entre los que se encuentra la Ang I (Cushman et al., 1981).

La Ang II actúa a través de dos receptores, el receptor de angiotensina tipo 1 (AT_1) y el receptor de angiotensina tipo 2 (AT_2) (Peach, 1977). La unión de Ang II al receptor AT_1 causa vasoconstricción y estimula la liberación de aldosterona para aumentar la retención de agua y sal en el riñón. Por otro lado, las acciones del receptor AT_2 son contrarias a

las del AT_1, ya que participa en la liberación de NO provocando vasodilatación (Majumder y Wu, 2014).

Por tanto, desde el punto de vista de la regulación de la PA, sobre el SRAA se puede actuar de cuatro formas diferentes: inhibiendo la renina, inhibiendo la ECA, bloqueando los receptores de la Ang II o bloqueando los receptores de la aldosterona. Así es como funcionan los fármacos que existen para el tratamiento de la HTA, como por ejemplo el aliskiren, inhibidor de la renina; el captopril, inhibidor de la ECA; el valsartan, bloqueante del receptor AT_1; y la espironolactona, bloqueante de los receptores de aldosterona.

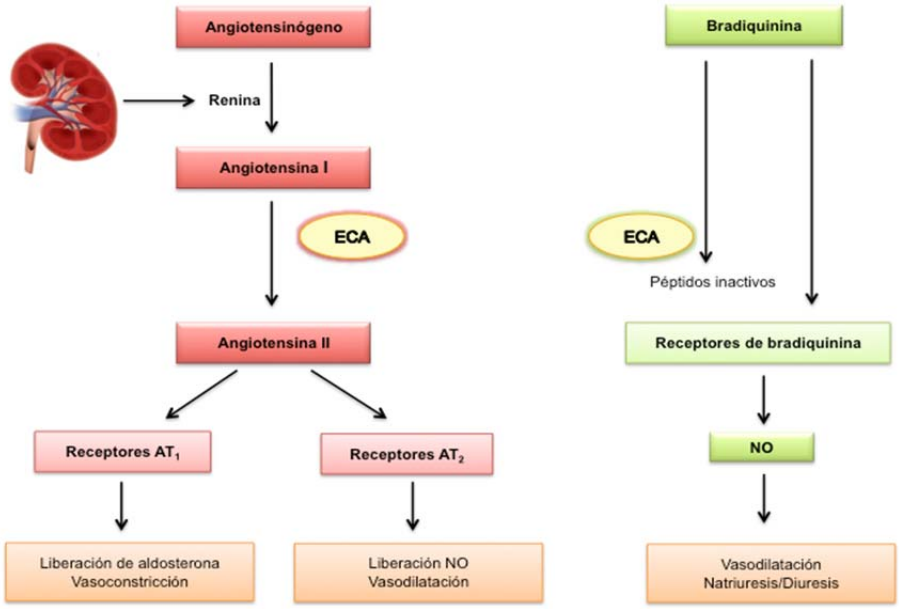

Figura 1. Sistema renina-angiotensina-aldosterona (SRAA) y sistema quinina-calicreína

1.2.2 Sistema quinina-calicreína

Las serin-proteasas denominadas calicreínas son el primer elemento de este sistema. Liberan bradiquinina, nonapéptido de potente acción vasodilatadora, a partir del quininógeno. La bradiquinina actúa a través de dos receptores, el tipo 1 (B_1) y el tipo 2 (B_2), que inducen la generación del compuesto vasodilatador NO en las células endoteliales. Además, los receptores tipo B_2 activan la fosfolipasa A2 que libera ácido araquidónico, lo que lleva a la formación de varios vasodilatadores, incluyendo la prostaciclina, que son antagonistas de la Ang II (Majumder y Wu, 2014). Como se muestra en la Figura 1, el SRAA y el sistema quinina-calicreína están conectados, ya que la ECA cataliza también la degradación de la bradiquinina y con ello, se produce un aumento de la PA.

1.2.3 Sistema óxido nítrico (NO)-endotelina (ET)

Las células endoteliales desempeñan importantes funciones fisiológicas en la regulación del equilibrio y la homeostasis vascular mediante la producción de sustancias vasoactivas, incluyendo el NO, vasodilatador, y la endotelina (ET), vasoconstrictora. Un desequilibrio entre estos dos factores es una característica típica de la disfunción endotelial (Majumder y Wu, 2014). El funcionamiento de este sistema se esquematiza en la Figura 2.

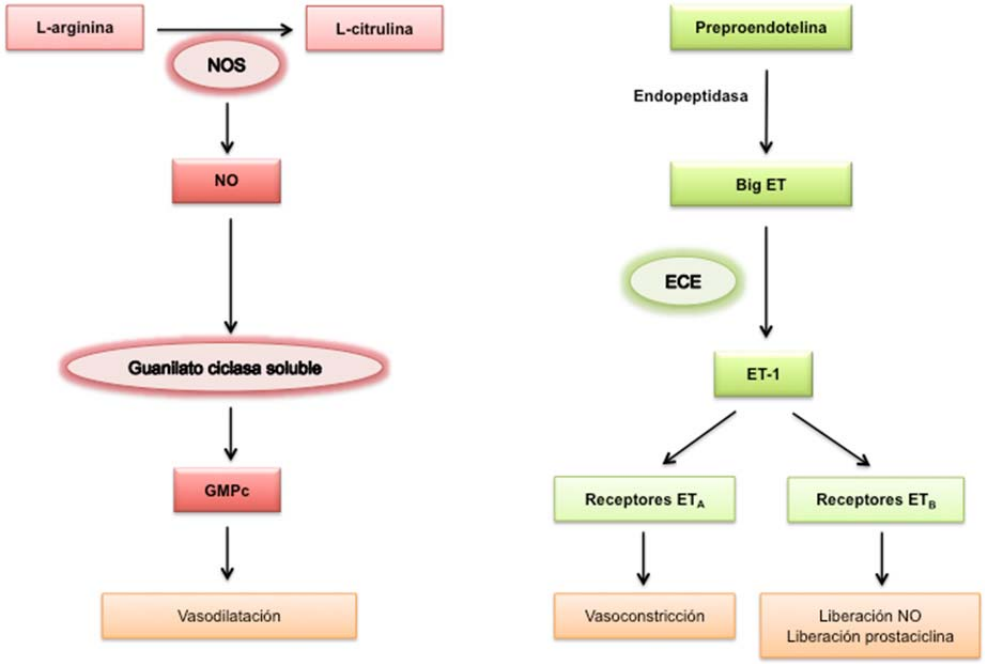

Figura 2. Sistema óxido nítrico (NO)-endotelina (ET)

El NO inicia y mantiene la vasodilatación a través de una serie de eventos biológicos. El NO es un gas de fácil difusión que se produce en las células endoteliales por medio de la enzima óxido nítrico sintasa (NOS), que transforma el aminoácido L-arginina en L-citrulina y NO. Se han identificado tres isoformas de la NOS que utilizan L-arginina como sustrato, la isoforma endotelial (eNOS), la neuronal (nNOS) y la inducible (iNOS) (Vural y Bayazit, 2001; Barbato y Tzeng, 2004). Una de las principales funciones del NO es regular el tono vascular mediante la activación de la guanilato ciclasa soluble (GCs). En las células del músculo liso vascular, la GCs aumenta las concentraciones intracelulares de guanosín monofosfato cíclico (GMPc), induciendo así la relajación (Rodríguez-Feo et al., 2001). Por ello, la disfunción endotelial está relacionada con la HTA.

Contrariamente, las ETs circulantes tienen propiedades vasoconstrictoras. El gen de la ET humana codifica para la preproendotelina, péptido de 212 aminoácidos, que bajo la acción de una endopeptidasa tipo furina se transforma en proendotelina o big ET, péptido de 38 aminoácidos sin actividad biológica. Esta última es una pre-hormona que es secretada y liberada a la circulación; la enzima conversora de endotelina (ECE) la transforma en endotelina-1 (ET-1), péptido de 21 aminoácidos con un potente efecto vasoconstrictor. Se han caracterizado tres isoformas de ETs (ET-1, ET-2 y ET-3), pero la ET-1 es la isoforma dominante y modula activamente las funciones vasculares. La mayor parte de la ET-1 se libera hacia las células musculares adyacentes al endotelio, donde se une a receptores específicos, que pueden ser tipo A (ET_A) o tipo B (ET_B). La unión de ET-1 con el receptor ET_A en el músculo liso vascular produce vasoconstricción, mientras que la unión de ET-1 con el receptor ET_B produce liberación de NO y prostaciclina (Barton y Yanagisawa, 2008). Los distintos componentes del sistema ET también son la diana de diferentes fármacos antihipertensivos, como por ejemplo el fosforamidón que es un inhibidor de la ECE o el bosentán, bloqueante de los receptores de ET (Schiffrin, 2005).

La interacción entre el NO y la ET-1 es importante en numerosas condiciones fisiopatológicas. La reducción en la biodisponibilidad de NO está asociada con un aumento en la expresión de la ET-1. Del mismo modo, el NO antagoniza la vía de la ET-1 a través de diferentes mecanismos (Bourque et al., 2011), por lo que estas relaciones sugieren un vínculo íntimo entre estos dos mediadores para mantener el equilibrio de la función endotelial y en consecuencia el tono vascular (Barton y Yanagisawa, 2008).

1.2.4 Sistema de los péptidos natriuréticos

El sistema de los péptidos natriuréticos se compone principalmente de tres péptidos, el péptido natriurético auricular, el péptido natriurético tipo B y el péptido natriurético tipo C. Las propiedades biológicas de estos tres péptidos incluyen entre otras, natriuresis, vasodilatación e inhibición de la secreción de renina, con lo que se inhibe el SRAA. Los péptidos natriuréticos se eliminan de la circulación a través de la degradación enzimática llevada a cabo por la endopeptidasa neutra. Esta enzima también es fundamental para el procesamiento y el catabolismo de la Ang I, la bradiquinina y la ET-1, así como muchos otros péptidos. Dado que muchos sustratos de la endopeptidasa neutra son sustancias vasoactivas y con acciones diuréticas/natriuréticas, los inhibidores de la endopeptidasa neutra se han examinado como tratamiento farmacológico de la HTA (Mangiafico et al., 2013).

2. Peptidos bioactivos con capacidad antihipertensiva

Los seres humanos ingieren un promedio de 50-70 g de proteínas al día. Al ingerir las proteínas, éstas son hidrolizadas a péptidos y aminoácidos, los cuales son absorbidos y transportados por la corriente sanguínea. Además de su función nutricional, algunos de estos péptidos comparten características estructurales con péptidos reguladores endógenos, por lo que pueden interaccionar con sus mismos receptores, enzimas y otras dianas moleculares para ejercer así una función reguladora. Estos péptidos de tamaño pequeño, entre 2 y 50 residuos aminoacídicos, son conocidos como péptidos bioactivos y se definen como secuencias de aminoácidos inactivas en el interior de la proteína precursora, pero con diversas actividades biológicas tras su liberación

mediante hidrólisis en el tracto gastrointestinal o durante el procesado de los alimentos (Meisel et al., 1989). Estas secuencias peptídicas despiertan un gran interés debido a la variedad de actividades biólogicas que pueden ejercer (actividad opiácea, inmunomoduladora, antimicrobiana, antitrombótica, quelante de minerales y antihipertensiva, entre otras) y a su multifuncionalidad.

Dentro de los péptidos bioactivos, aquellos con efecto antihipertensivo han sido con diferencia los más estudiados, debido a que el uso prolongado de fármacos para el tratamiento de la HTA suele producir efectos secundarios como cuadros de hipotensión y de tos, pérdida del sentido del gusto, fatiga, erupciones cutáneas e incluso malformaciones congénitas (Atkinson y Robertson, 1979). Por ello, en las últimas décadas se ha puesto de manifiesto un considerable interés en potenciar el empleo de componentes naturales de los alimentos para tratar la HTA en aquellos pacientes con PA normal-alta y con hipertensión en grado leve o moderado. En este sentido, la ingesta de una dieta rica en proteínas parece tener un efecto favorable sobre la PA en individuos hipertensos debido a la generación de péptidos antihipertensivos durante la digestión proteolítica en el tracto gastrointestinal (Hong et al., 2008; Wang et al., 2008).

La metodología que se emplea para caracterizar los diferentes péptidos antihipertensivos consiste en realizar primero estudios *in vitro* de inhibición de la ECA seguido de estudios *in vivo* en modelos animales. Los estudios *in vitro* sirven de cribado previo para seleccionar péptidos que pudieran tener actividad antihipertensiva *in vivo.* Para realizar estos estudios se han desarrollado métodos analíticos rápidos, sensibles y fiables, basados generalmente en ensayos espectrofotométricos y cromatográficos (Cushman y Cheung, 1971; Wu et al., 2002; Vermeirssen et al., 2004; Li et al., 2005; Sentandreu y Toldrá, 2006), los cuales

determinan la actividad de la 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 la evaluación *in vivo* se emplean ratas espontáneamente hipertensas (SHRs) que son el modelo de hipertensión comúnmente aceptado por la comunidad científica (FitzGerald et al., 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 PA de ratas normotensas Wistar-Kyoto (WKY).

Sin embargo, estos ensayos en su conjunto han puesto de manifiesto una falta de correlación importante entre la potencia inhibitoria *in vitro* de la ECA y el efecto *in vivo.* Este hecho, pone en duda la utilización de los datos *in vitro* como único criterio de selección de péptidos con potencial antihipertensivo, dado que los péptidos son susceptibles de sufrir degradación tras su administración oral hasta alcanzar su órgano diana, y también por la posibilidad de que intervengan otros mecanismos de acción diferentes a la inhibición de la ECA.

Un paso más allá de la caracterización *in vitro* de la inhibición de la ECA son los ensayos funcionales *ex vivo* empleando arterias aisladas. Este tipo de estudios registran efectos vasoactivos y aportan evidencia funcional de la capacidad de los péptidos para inhibir la vasoconstricción ECA-dependiente (Centeno et al., 2006) o para ejercer efectos vasoactivos a través de otros mecanismos de acción al margen de la ECA. Además, también se puede demostrar la inhibición *in vivo* de la ECA determinando los niveles en plasma y/o tejido no sólo de la enzima, sino también del resto de componentes del SRAA, aunque todavía hay pocos estudios de este tipo (Wang et al., 2008; Fernández-Musoles et al., 2013b; Koyama et

al., 2014). Asimismo, empiezan a ser frecuentes los estudios que abordan el efecto de péptidos e hidrolizados en la expresión génica de los componentes del SRAA (Li et al., 2011; Lu et al., 2011; Yu et al., 2014).

Se han aislado péptidos antihipertensivos a partir de fuentes proteicas muy diversas, tanto animales como vegetales: el huevo, la carne, diferentes vegetales y animales marinos (Martínez-Maqueda et al., 2012). No obstante, dentro de los alimentos, las proteínas lácteas siguen siendo una de las fuentes principales de péptidos antihipertensivos (Murray y FitzGerald, 2007).

2.1 La leche como fuente de péptidos antihipertensivos

La leche está compuesta mayoritariamente por agua, hidratos de carbono, proteínas y lípidos, aunque también existen otros componentes minoritarios como son enzimas, vitaminas y sales minerales. Las proteínas se encuentran en una proporción de 30-35 g/L y constituyen la fracción más compleja de la leche. En la leche existen dos tipos de proteínas, las caseínas (CN), que constituyen un 80% del contenido proteico, y las proteínas del suero (Whitney, 1988). Las proteínas mayoritarias del suero son la β-lactoglobulina (β-Lg), la α-lactoalbúmina (α-La), la seroalbúmina (BSA) y las inmunoglobulinas (Ig) (Fox y Flynn, 1994). Las proteínas minoritarias incluyen la lactoferrina (LF) y distintas enzimas, como la plasmina y la lactoperoxidasa (Walstra y Jenness, 1984).

Los estudios realizados hasta la fecha sugieren que el consumo de leche y productos lácteos está inversamente relacionado con el riesgo de padecer hipertensión. Estos productos son ricos en minerales, que podrían reducir la PA. Sin embargo, las proteínas y los péptidos bioactivos

provenientes de éstas parecen ser los principales responsables del efecto antihipertensivo (Jauhiainen y Korpela, 2007).

La mayor parte de los péptidos antihipertensivos generados a partir de las proteínas lácteas provienen de la CN, y son inhibidores *in vitro* de la ECA. La relación entre secuencia peptídica y potencia inhibitoria de estos péptidos no está completamente definida, ya que también es importante la longitud del péptido y su potencial electrostático (Li et al., 2005; De Leo et al., 2009). En general, los péptidos inhibidores de la 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 (Ala, Leu, Ile, Val, Pro, Phe y Met). Además, muchos de estos inhibidores contienen residuos de Lys o Arg en este mismo extremo, lo que parece sugerir que la carga positiva de estos aminoácidos contribuye a la potencia inhibitoria de la ECA (Murray y FitzGerald, 2007). En concreto, se ha descrito que la presencia de un residuo de Pro en antepenúltima posición favorece la unión del péptido inhibidor al centro activo de la ECA (Rohrbach et al., 1981), así como la importancia de un residuo de Leu en el extremo C-terminal para aumentar la potencia inhibitoria de los péptidos (Gomez-Ruiz et al., 2004).

También se han usado técnicas como el acoplamiento molecular para predecir secuencias peptídicas inhibidoras de la ECA (Norris et al., 2012; Hai-Bang y Shimizu, 2014). Dipéptidos que contienen Trp como AW (α_{S1}-CN f(163-164)), IW (α-La f(59-60)) y VW (LF f(267-268) y f(346-347)) han demostrado ser inhibidores competitivos de la ECA humana. Además, la localización de un residuo de Trp en la posición C-terminal de dipéptidos como RW y VW se ha relacionado con una mayor inhibición de la ECA en comparación con los péptidos de secuencia inversa (WR y WV). Estas diferencias se han explicado por el hecho de que se produzcan mayores

interacciones hidrofóbicas con el sitio activo de la ECA cuando el residuo de Trp se encuentra en la posición C-terminal (Lunow et al., 2015; Nongonierma y FitzGerald, 2015).

2.2 Estrategias para la producción de péptidos antihipertensivos a partir de proteínas lácteas

Los péptidos antihipertensivos integrados en las secuencias de las proteínas alimentarias pueden ser liberados durante el proceso digestivo o producidos mediante hidrólisis enzimática por la acción de enzimas proteolíticas exógenas, o a través de la fermentación por microorganismos proteolíticos como las bacterias ácido lácticas (BAL) (López-Fandiño et al., 2006). Ambas estrategias de producción se han aplicado con éxito y han permitido la obtención de péptidos inhibidores de la ECA con efecto antihipertensivo en modelos animales y en ensayos clínicos, como se describe a continuación.

2.2.1 Hidrólisis enzimática

La hidrólisis enzimática ha sido la estrategia más común para la obtención de péptidos bioactivos a partir de proteínas alimentarias. Se realiza empleando enzimas implicadas en la digestión gastrointestinal (pepsina gástrica y tripsina pancreática), simulando las condiciones fisiológicas (pH y temperatura) de la digestión *in vivo*. También se han empleado enzimas de origen vegetal como la bromelaína, y de origen microbiano como la proteinasa K, la termolisina y proteasas de la pared celular de BAL (López-Fandiño et al., 2006).

La hidrólisis enzimática permite seleccionar la proteína a hidrolizar, la enzima y las condiciones del proceso (temperatura, pH y tiempo), por lo que se consigue optimizar la hidrólisis. Además, se puede mejorar el proceso empleando métodos de enriquecimiento de las fracciones y de aislamiento de los péptidos bioactivos, para lo que se utilizan técnicas de precipitación, ultrafiltración y cromatografía. Los péptidos inhibidores de la ECA suelen tener tamaños menores a 3 kDa, por lo que se emplean técnicas como la ultrafiltración o la cromatografía de exclusión molecular (López-Fandiño et al., 2006).

Teniendo en cuenta las características de estructura-actividad de los péptidos inhibidores de la ECA, enzimas con especificidad para liberar péptidos con un extremo C-terminal hidrofóbico, o con residuos de Lys o Arg podrían ser muy útiles. Además, la elección del mejor binomio proteína-enzima puede ser realizada gracias al desarrollo de cálculos *in silico* (Vermeirssen et al., 2004). Estos cálculos indican que además de la CN, la LF puede ser una buena proteína precursora de péptidos inhibidores de la ECA, dado que posee en distintas zonas de su secuencia el tripéptido LRP con un valor de IC_{50} de 0,27 μM (Vermeirssen et al., 2004).

A pesar de que un gran número de péptidos producidos mediante hidrólisis enzimática de proteínas lácteas, mayoritariamente CN, muestran actividad inhibidora de la ECA *in vitro*, sólo una pequeña proporción de ellos presenta efecto antihipertensivo *in vivo* en SHRs (Tabla 1).

Tabla 1. Péptidos derivados de proteínas lácteas, inhibidores de la ECA y con efecto antihipertensivo en SHRs, obtenidos mediante hidrólisis enzimática (adaptada de Martínez-Maqueda et al., 2012)

Enzima	Fragmento	Secuencia	IC_{50} (μM)	Dosis (mg/kg)	Descenso PAS (mmHg)	Referencia
Tripsina	α_{S1}-CN f(23-34)	FFVAPFPGVFGK	77,0	100,0	34,0	(Karaki et al., 1990)
	α_{S1}-CN f(194-199)	TTMPLW	16,0	100,0	13,6	(Karaki et al., 1990)
	β-CN f(177-183)	AVPYPQR	15,0	100,0	10,0	(Karaki et al., 1990)
	κ-CN f(106-112)	MAIPPKK	4785,0	10,0	28,0	(Miguel et al., 2007)
Pepsina	α_{S1}-CN f(90-94)	RYLGY	0,7	5,0	25,0	(Contreras et al., 2009)
	α_{S1}-CN f(143-149)	AYFYPEL	6,6	5,0	21,0	(Contreras et al., 2009)
	α_{S2}-CN f(89-95)	YQKFPQY	20,1	5,0	15,0	(Contreras et al., 2009)
	α_{S2}-CN f(203-208)	PYVRYL	1,9	3,0	23,4	(Recio et al., 2005)
Proteinasa K	β-CN f(59-61)	VYP	288,0	8,0	21,0	(Abubakar et al., 1998)
	β-CN f(59-64)	VYPFPG	221,0	8,0	22,0	(Abubakar et al., 1998)
	β-CN f(80-90)	TPVVVPPFLQP	749,0	8,0	8,0	(Abubakar et al., 1998)
	β-Lg f(78-80)	IPA	141,0	8,0	31,0	(Abubakar et al., 1998)
	BSA f(221-222)	FP	315,0	8,0	27,0	(Abubakar et al., 1998)
Termolisina	β-Lg f(58-61)	LQKW	34,7	10,0	18,1	(Hernández-Ledesma et al., 2007)
	β-Lg f(103-105)	LLF	79,8	10,0	29,0	(Hernández-Ledesma et al., 2007)
Pepsina, quimotripsina y tripsina	κ-CN f(22-24)	IAK	15,7	4,0	20,7	(Miguel et al., 2010)
	κ-CN f(61-66)	YAKPVA	14,3	6,0	23,1	(Miguel et al., 2010)
	κ-CN f(76-86)	WQVLPNAVPAK	10,1	7,0	18,4	(Miguel et al., 2010)
	κ-CN f(98-105)	HPHPHLSF	28,9	10,0	15,7	(Miguel et al., 2010)

Tabla 1 (continuación)

Enzima	Fragmento	Secuencia	IC_{50} (μM)	Dosis (mg/kg)	Descenso PAS (mmHg)	Referencia
Pancreáticas y gástricas	α-La f(50-53)	YGLF	733,0	0,1	23,4	(Nurminen et al., 2000)
Proteinasa de *Lactobacillus helveticus* CP790	α_{S1}-CN f(104-109)	YKVPQL	22,0	2,0	13,0	(Maeno et al., 1996)
	α_{S2}-CN f(189-192)	AMPKPW	580,0	2,0	5,0	(Maeno et al., 1996)
	α_{S2}-CN f(190-197)	MKPWIQPK	300,0	2,0	3,0	(Maeno et al., 1996)
	α_{S2}-CN f(198-202)	TKVIP	400,0	2,0	9,0	(Maeno et al., 1996)
	β-CN f(140-143)	LQSW	500,0	2,0	2,0	(Maeno et al., 1996)
	β-CN f(169-174)	KVLPVP	5,0	2,0	32,2	(Maeno et al., 1996)
	β-CN f(169-175)	KVLPVPQ	1000,0	2,0	31,5	(Maeno et al., 1996)
Corolasa PP	β-CN f(134-138)	HLPLP	21	7,0	23,5	(Hernández-Ledesma et al., 2007; Miguel et al., 2006; Quirós et al., 2007; Quirós et al., 2012)
Proteinasa de *Aspergillus oryzae*	β-CN f(74-76)	IPP	5,0	0,3	28,3	(Mizuno et al., 2004; Nakamura et al., 1995a; Nakamura et al., 1995b)
	β-CN f(84-86)	VPP	9,0	0,6	32,1	(Mizuno et al., 2004; Nakamura et al., 1995a; Nakamura et al., 1995b)

2.2.2 Procesos fermentativos

La fermentación láctica es otro de los procesos que se ha empleado para la producción de péptidos con actividad inhibidora de la ECA y efecto antihipertensivo. Para que este proceso se produzca con éxito es determinante el cultivo iniciador empleado, principalmente su capacidad proteolítica y la especificidad de sus enzimas (Hernández-Ledesma et al., 2004).

2.2.2.1 Bacterias ácido lácticas (BAL)

Las BAL tienen las ventajas de asimilar la lactosa, poseer un sistema proteolítico potente y tener carácter probiótico. Se ha demostrado que ciertas especies de BAL como *Lactobacillus helveticus*, *Lb. rhamnosus*, *Lb. acidophilus* o *Lactococcus lactis* subs*. cremoris* son capaces de producir leches fermentadas que contienen péptidos inhibidores de la ECA (Tabla 2) (Leclerc et al., 2002; Hernández-Ledesma et al., 2004). *Lb. helveticus* tiene mayor actividad proteolítica que otras especies, y muchos de los péptidos antihipertensivos derivados de CN se han obtenido fermentando la leche con esta bacteria. Un ejemplo es el producto lácteo comercializado en Japón por la empresa Calpis (Calpis/AmealS®, Calpis Co. Ltd., Tokio, Japón), fermentado con *Lb. helveticus* y *Saccharomyces cerevisiae,* y que contiene los dos potentes péptidos VPP e IPP, conocidos como lactotripéptidos (Korhonen, 2009). Se ha demostrado su efecto antihipertensivo en SHRs después de su administración aguda, y posteriormente un estudio doble ciego mostró una reducción significativa de la PAS y de la PAD en pacientes hipertensos con y sin medicación (Nakamura et al., 1995a; Nakamura et al., 1995b; Masuda et al., 1996). Esta leche fermentada también se comercializó en Finlandia (Valio Evolus® Double Effect, Valio Ltd., Finlandia). Es importante destacar

que estos dos lactotripéptidos derivados de CN también pueden ser producidos mediante hidrólisis enzimática utilizando una preparación proteolítica de *Aspergillus oryzae* (Mizuno et al., 2004).

Otra especie bacteriana capaz de liberar péptidos con capacidad inhibitoria de la ECA y efecto antihipertensivo en SHRs a partir de la β-caseína es *Enterococcus faecalis* (Tabla 2) (Muguerza et al., 2006; Quirós et al., 2007). En concreto, se identificó la secuencia LHLPLP como uno de los péptidos responsables del efecto antihipertensivo provocado por una leche fermentada por *E. faecalis.* Estudios posteriores demostraron que el pentapéptido HLPLP era el fragmento mínimo activo responsable del efecto antihipertensivo de LHLPLP (Quirós et al., 2008). Dado el potencial antihipertensivo de estas secuencias, se optimizó la producción de HLPLP mediante hidrólisis enzimática con enzimas de grado alimentario (Tabla 1) (Quirós et al., 2012).

Tabla 2. Péptidos derivados de proteínas lácteas, inhibidores de la ECA y con efecto antihipertensivo en SHRs, obtenidos mediante procesos fermentativos con BAL (adaptada de Martínez-Maqueda et al., 2012)

Microorganismos	Fragmento	Secuencia	IC_{50} (μM)	Dosis (mg/kg)	Descenso PAS (mmHg)	Referencia
Lb. helveticus CPN4	α_{S1}-CN f(146-147)	YP	720	2,0	-32,1	(Yamamoto y Takano, 1999)
Lb. helveticus y *S. cerevisiae*	β-CN f(74-76)	IPP	5,0	0,3	-28,3	(Nakamura et al., 1995a; Nakamura et al., 1995b)
	β-CN f(84-86)	VPP	9,0	0,6	-32,1	(Nakamura et al., 1995a; Nakamura et al., 1995b)
E. faecalis	β-CN f(58-76)	LVYPFPGPIPNSL-PQNIPP	5,2	6,0	-14,9	(Miguel et al., 2006; Quirós et al., 2007)
	β-CN f(133-138)	LHLPLP	5,5	3,0	-25,3	(Miguel et al., 2006; Quirós et al., 2007)
	β-CN f(133-139)	LHLPLPL	425,0	10,0	-7,7	(Miguel et al., 2006; Quirós et al., 2007)
	β-CN f(134-138)	HLPLP	21,0	7,0	-23,5	(Miguel et al., 2006: Hernández-Ledesma et al., 2007; Quirós et al., 2007)
	β-CN f(197-206)	VLGPVRGPFP	137,0	10,0	-16,2	(Miguel et al., 2006; Quirós et al., 2007)
	β-CN f(201-209)	VRGPFPIIV	599,0	10,0	-16,1	(Miguel et al., 2006; Quirós et al., 2007)

2.2.2.2 Levaduras no convencionales

Las levaduras se han empleado tradicionalmente en la fabricación de diferentes alimentos y bebidas. El mejor ejemplo es *S. cerevisiae*, la principal levadura que participa en el proceso de elaboración de cerveza, vino y pan. Sin embargo, en las últimas décadas otras especies conocidas como levaduras no convencionales o levaduras no-*Saccharomyces* se están empleando como herramientas biotecnológicas, no sólo para la fabricación de alimentos y bebidas, sino también en diferentes procesos de investigación biomédica, en biocatálisis y en la producción de enzimas, hormonas y vacunas (Johnson, 2013).

Las especies de levaduras no convencionales *Debaryomyces hansenii*, *Kluyveromyces lactis* y *Kluyveromyces marxianus*, *Yarrowia lipolytica* así como distintas especies de los géneros *Candida* y *Pichia* juegan un papel clave en los procesos de fabricación de productos lácteos (De Freitas et al., 2009). Estas levaduras suscitan también interés por su potencial proteolítico, en algunos casos similar al de las BAL (Klein et al., 2002; Bintsis et al., 2003) y por su posible carácter probiótico (Kumura et al., 2004; Maccaferri et al., 2012; Pedersen et al., 2012; Smith et al., 2014). Además, *K. lactis, K. marxianus* y *D. hansenii* son consideradas GRAS (Generally Recognized As Safe), por lo que pueden utilizarse en la industria alimentaria.

Las levaduras pertenecientes al género *Kluyveromyces* presentan una alta variabilidad en sus características morfológicas, fisiológicas y moleculares, lo que explica su naturaleza ubicua. *K. lactis* y *K. marxianus* son capaces de fermentar y asimilar la lactosa, y se encuentran en diferentes productos lácteos como mantequilla, queso, leche, yogur y kéfir (Büchl y Seiler, 2011). Debido a su capacidad de fermentar la lactosa, así como sus actividades lipolíticas y proteolíticas, el potencial biotecnológico

de este género ha sido extensamente estudiado (van Ooyen et al., 2006; Fonseca et al., 2008), destacando la producción de bioingredientes a partir del suero de leche (Belem y Lee, 1998). Por lo que se refiere a la producción de péptidos, se ha descrito la combinación de *K. marxianus* con *Lb. rhamnosus* para lograr fermentados lácteos con actividad inhibidora *in vitro* de la ECA (Hamme et al., 2009). Además, una cepa de *K. marxianus* aislada de Kumis colombiano generó una leche fermentada con bajo contenido alcohólico y actividad inhibidora *in vitro* de la ECA (Chaves-López et al., 2012).

En cuanto al género *Debaryomyces*, es capaz de crecer en ambientes con baja actividad de agua, en el suelo, en el agua del mar y en diferentes alimentos como la leche y la carne fermentada, productos en salmuera y diferentes bebidas alcohólicas (Zhang et al., 2012). *D. hansenii* suele ser la especie predominante en quesos de distintos orígenes (Padilla et al., 2014; Padilla, 2014), y se ha demostrado su capacidad para hidrolizar la CN (Kumura et al., 2002), aunque no se ha estudiado su potencial para producir péptidos bioactivos.

También se ha descrito la producción de péptidos con actividad inhibidora *in vitro* de la ECA a partir de α-La (WLAHK) por el género *Candida* (Didelot et al., 2006a; Didelot et al., 2006b). Sin embargo, es interesante remarcar que el efecto antihipertensivo *in vivo* de las secuencias peptídicas o leches fermentadas generadas por levaduras no ha sido demostrado.

2.3 Efecto antihipertensivo en estudios clínicos

Para demostrar los efectos beneficiosos de los péptidos antihipertensivos, una vez realizados los ensayos preclínicos oportunos en animales de experimentación, el siguiente paso son los ensayos clínicos en humanos. Algunas de las secuencias detalladas en las Tablas 1 y 2 han sido evaluadas en este tipo de ensayos, si bien la mayoría de los ensayos clínicos realizados hasta la fecha se han centrado en los lactotripéptidos VPP e IPP (Boelsma y Kloek, 2009; Usinger et al., 2009). La mayoría de estos estudios concluyen que la administración de los lactotripéptidos, en dosis que varían entre 3 y 52 mg/kg, provoca un descenso de la PAS y de la PAD. Sin embargo, otros estudios aportan resultados contradictorios al indicar que estos péptidos no causan variaciones en la PA de sujetos holandeses y daneses (Engberink et al., 2008; Usinger et al., 2010). Se ha propuesto que la genética y los hábitos alimentarios podrían explicar estos resultados contradictorios. De hecho, en un estudio reciente, se demuestra que el efecto antihipertensivo de los lactotripéptidos fue mayor en individuos asiáticos que caucásicos (Cicero et al., 2011), lo que puede deberse a polimorfismos que afectarían a la eficacia de estos péptidos, al igual que ocurre con ciertos fármacos (Arsenault et al., 2010). Estos estudios también han puesto de manifiesto la implicación de otros mecanismos de acción además de la inhibición de la ECA. Por ejemplo, en un estudio realizado con 70 sujetos hipertensos de raza caucásica, al administrar VPP e IPP se observaba un descenso de la PA, pero no se observaba variación en la actividad de la renina plasmática o en los niveles de la Ang I y de la Ang II (Yamasue et al., 2010). Estos resultados contradictorios impulsaron a la EFSA a emitir un informe indicando que existe falta de evidencia suficiente que permita demostrar el mecanismo por el cual los lactotripéptidos VPP e IPP pueden contribuir a mantener una

PA adecuada en humanos (Agostoni et al., 2012). Finalmente, en un meta-análisis reciente que recoge 19 estudios clínicos llevados a cabo con dosis pequeñas de los lactotripéptidos, se muestra un efecto global sobre la PA (-4,0 mm Hg para la PAS; -1,0 mm Hg para la PAD) aunque el efecto positivo no se reflejó en todos los ensayos (Turpeinen et al., 2013). Además, en otro meta-análisis realizado por Cicero et al., (2013), se observó la relación inversa entre la edad de los sujetos participantes en los ensayos y el efecto sobre la PAS provocado por la administración de los lactotripeptidos.

Además de los lactotripéptidos, se han evaluado otros péptidos en ensayos clínicos. Por ejemplo, un yogur enriquecido con un hidrolizado de CN conteniendo las secuencias RYLGY y AYFYPEL, fue evaluado en 71 sujetos hipertensos y 50 voluntarios normotensos. Después de 6 semanas de consumo del yogur, se observó un descenso de la PAS de 12,5 mm Hg en los pacientes hipertensos, mientras que no se detectaron cambios significativos en la PA del grupo normotenso (Recio et al., 2011). Asimismo, también se han descrito efectos positivos para péptidos de origen no lácteo, aunque este tipo de estudios son todavía escasos (Bouglé y Bouhallab, 2015).

2.4 Lactoferrina como fuente de péptidos antihipertensivos

La lactoferrina (LF) es una glicoproteína de 80 kDa de la familia de las transferrinas (Wakabayashi et al., 2006) aislada por primera vez como una 'fracción roja' de la leche de vaca (Sorensen y Sorensen, 1939). Tiene dos lóbulos, correspondientes a los extremos amino terminal (N-terminal) (residuos 1-333) y C-terminal (residuos 345-692), que son homólogos en un 37% y están conectados por una cadena α-hélice (residuos 334-344).

Cada uno de estos lóbulos está subdividido en dos dominios, que poseen una zona para unión reversible de hierro. Cada átomo de hierro está coordinado a cuatro ligandos (dos residuos de Tyr, un residuo de Asp y un residuo de His), además de a un anión, habitualmente carbonato (Farnaud y Evans, 2003).

La LF se localiza en superficies mucosas, en el interior de gránulos específicos de leucocitos polimorfonucleares y, no solo en la leche, sino también en otras secreciones como la saliva y el fluido seminal, pudiendo jugar un papel protector en la respuesta inmune innata (Farnaud y Evans, 2003). Además, presenta una amplia gama de actividades biológicas, entre las que se incluyen la antimicrobiana, antiviral, antioxidante, inmunomoduladora y moduladora del crecimiento celular (Wakabayashi et al., 2006; Jenssen y Hancock, 2009).

La leche es la principal fuente de LF, cuya concentración varía en función de la especie, encontrándose en cantidades mayores en la leche humana que en la de vaca. Aunque es una proteína minoritaria del suero lácteo, hace casi cuarenta años que se desarrolló la tecnología para producir grandes cantidades de LF de alta pureza a partir de leche de vaca (Law y Reiter, 1977). También se puede obtener LF mediante tecnología de DNA recombinante en microorganismos, plantas o ganado bovino (Wakabayashi et al., 2006). Es evidente que la administración oral de LF ejerce efectos beneficiosos sobre la salud de los seres humanos y de los animales, justificando así su potencial aplicación como aditivo alimentario (Farnaud y Evans, 2003; Wakabayashi et al., 2006; Tomita et al., 2009; Ammons y Copie, 2013). Por lo que se refiere a los efectos de la LF sobre la PA, se ha descrito que su administración crónica la reduce, y además mejora la capacidad antioxidante en un modelo de hipertensión inducida con dexametasona en rata, lo que sugiere la participación del efecto antioxidante en el mecanismo de acción antihipertensivo de la LF

(Safaeian y Zabolian, 2014). Además, la LF posee un efecto antihipertensivo dependiente de NO, que podría estar mediado por el sistema central opiodérgico (Hayashida et al., 2004).

Respecto a péptidos derivados de LF, en un trabajo pionero sobre la estabilidad de proteínas antimicrobianas en el tracto digestivo, se demostró que mediante hidrólisis de la LF bovina empleando pepsina, se generaba un péptido, la lactoferricina bovina (LfcinB) con mayor actividad antibacteriana que la proteína nativa (Bellamy et al., 1992). Este péptido, además de la actividad antibacteriana, también tiene otras actividades biológicas que incluyen la antifúngica, la antiviral y la antitumoral (Eliassen et al., 2002; Andersen et al., 2004; Muñoz y Marcos, 2006). Sin embargo, aunque en 2004 se indicó que la LF podía ser una buena proteína precursora de péptidos inhibidores de la ECA (Vermeirssen et al., 2004), se han realizado pocas investigaciones acerca de hidrolizados o péptidos antihipertensivos derivados de la misma. En 2006 se describió la secuencia LRPVAA con efecto antihipertensivo en SHRs tras administración intravenosa (Lee et al., 2006) y, desde entonces, nuestro grupo de investigación ha caracterizado hidrolizados y péptidos derivados de LF con capacidad inhibitoria *in vitro* de la ECA, efectos inhibitorios sobre la vasoconstricción ECA-dependiente, y efectos antihipertensivos en SHRs tras administración oral, tal y como se refleja en la Tabla 3.

Tabla 3. Péptidos e hidrolizados derivados de LF con potencia inhibitoria *in vitro* de la ECA, efectos inhibitorios sobre la vasoconstricción ECA-dependiente y efectos antihipertensivos en SHRs (adaptada de Manzanares et al., 2015).

Péptido	Secuencia	IC_{50}[1]	Inhibición de la vasoconstricción (%)[2]	PAS (mm Hg)[3]
Derivados de LfcinB				
$LfcinB_{17-32}$	FKCRRWQWRMKKLGAP	11,0 ± 1,5	20	n.s.
$LfcinB_{17-31}$	FKCRRWQWRMKKLGA	25,5 ± 2,3	21	n.s.
$LfcinB_{20-25}$	RRWQWR	32,0 ± 4,9	30	16,7 ± 3,2
$LfcinB_{19-25}$	CRRWQWR	2,3 ± 0,1	n.s.	n.s.
$LfcinB_{18-25}$	KCRRWQWR	5,8 ± 0,2	25	n.s.
$LfcinB_{17-25}$	FKCRRWQWR	2,9 ± 0,6	26	n.s.
$LfcinB_{17-24}$	FKCRRWQW	10,5 ± 0,6	18	n.s.
$LfcinB_{17-22}$	FKCRRW	26,7 ± 1,9	28	n.s.
$LfcinB_{21-23}$	RWQ	n.d.	n.d.	n.s.
$LfcinB_{22-23}$	WQ	n.d.	n.s.	11,4 ± 2,7
Derivados de LF				
f(266-270)	LIWKL	0,47 ± 0,01	22	25,3 ± 3,5
f(133-136)	RPYL	56,5 ± 1,9	14	18,9 ± 2,3
f(232-238)	LNNSRAP	105,3 ± 6,4	n.s.	15,3 ± 3,7
Hidrolizados enzimáticos de LF (LFH)				
Pepsina LFH		14,3 ± 3,3	29	15,9 ± 3,6
Proteinasa K LFH		1,3 ± 0,1	n.s.	19 ± 7
Tripsina LFH		6,9 ± 0,2	n.d.	n.s.

[1]Los valores de IC_{50} son expresados en µM (péptidos) o µg/mL (hidrolizados).
[2]Inhibición de la vasoconstricción ECA-dependiente expresado como porcentaje respecto al control.
[3]Máximos descensos de la PAS después de las dosis orales de 10 mg/kg (péptidos) o 200 mg/kg (hidrolizados).
n.s. no significativo; n.d. no determinado.

Las secuencias caracterizadas derivan de diferentes regiones de la LF, incluido su dominio antimicrobiano LfcinB. Entre los péptidos derivados de LfcinB destaca $LfcinB_{20-25}$ (RRWQWR), el único que provocó efecto antihipertensivo en SHRs tras su administración oral, a pesar de que todos ellos mostraron potencia inhibitoria *in vitro* de la ECA e inhibición de la vasoconstricción ECA-dependiente, lo que indicaría una escasa biodisponibilidad (Ruiz-Gimenez et al., 2010). Los péptidos con efecto antihipertensivo LIWKL, RPYL y LNNSRAP fueron identificados a partir de un hidrolizado de LF generado con pepsina, cuyo efecto *in vivo* también fue demostrado tras su administración aguda a SHRs (Ruiz-Giménez et al., 2012). Asimismo, también se caracterizaron otros hidrolizados enzimáticos de LF utilizando proteinasa K y tripsina, aunque sólo el primero mostró efecto antihipertensivo en SHRs (Fernández-Musoles et al., 2013a). Cabe destacar que algunas secuencias (WQ, LNNSRAP) y el hidrolizado de LF producido con proteinasa K con efecto *in vivo*, no mostraron efecto inhibitorio sobre la vasoconstricción ECA-dependiente. Por lo tanto, su efecto antihipertensivo no puede explicarse a través de un efecto inhibidor de la actividad de la ECA y sugiere la participación de otros mecanismos de acción.

La inhibición de la actividad de la ECA parece ser el mecanismo de acción responsable, al menos en parte, de los efectos antihipertensivos del hidrolizado de LF obtenido con pepsina (Tabla 3). Con el objetivo de confirmar el mecanismo de acción *in vivo*, se evaluó el efecto de la ingesta crónica del hidrolizado sobre la progresión de la HTA en SHRs y sobre los componentes sanguíneos del SRAA. Los resultados indicaron que la administración crónica del hidrolizado atenuó e incluso revirtió la progresión de la hipertensión, y provocó una reducción de la actividad de la ECA circulante y de los niveles de Ang II y aldosterona, además de un incremento compensatorio de la actividad renina. Estos datos por tanto

apoyan la inhibición de la ECA como el mecanismo de acción *in vivo* responsable del efecto antihipertensivo del hidrolizado de LF en SHRs (Fernández-Musoles et al., 2013b).

2.5 Biodisponibilidad

La escasa biodisponibilidad de los péptidos antihipertensivos tras su administración oral puede explicar las discrepancias entre la capacidad inhibidora *in vitro* de la ECA y la capacidad antihipertensiva *in vivo*. Por el contrario, también debe tenerse en cuenta que la degradación gastrointestinal o plasmática de los péptidos puede originar secuencias con mayor potencia inhibitoria de la ECA y mayor efecto antihipertensivo (FitzGerald et al., 2004).

El efecto de un péptido antihipertensivo depende de su capacidad para alcanzar el órgano diana en su forma activa, ya que tras su administración oral va a estar sometido a una serie de procesos de degradación hasta poder llegar a desarrollar su actividad final (Vermeirssen et al., 2004). La digestión de péptidos grandes comienza en el estómago por la acción de la pepsina a pH ácido. En el intestino delgado, donde las condiciones son más alcalinas, los péptidos se degradan a otros más pequeños por la acción de las proteasas pancreáticas (tripsina, α-quimotripsina, elastasa y carboxipeptidasas A y B) (Hayes et al., 2007). Posteriormente, los péptidos son hidrolizados por las peptidasas de las microvellosidades intestinales y las peptidasas citoplasmáticas. Estos péptidos y aminoácidos son absorbidos por los enterocitos a través de un transporte paracelular, por difusión pasiva y endocitosis (Vermeirssen et al., 2003). Una vez que los péptidos alcanzan el torrente sanguíneo, son susceptibles a la acción proteolítica de un gran

número de peptidasas plasmáticas y del endotelio vascular, por lo que, para la mayoría de los péptidos el tiempo de vida medio en plasma queda limitado a minutos.

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. Los pocos estudios *in vivo* que abordan la biodisponibilidad de péptidos antihipertensivos, han demostrado que las concentraciones detectadas en plasma se mueven en el rango nanomolar y picomolar, como es el caso de las secuencias VPP e IPP, cuyo tiempo de vida medio de absorción y eliminación en plasma de cerdos fue máximo a los 5 y 15 minutos respectivamente (van der Pijl et al., 2008). Recientemente, se ha investigado la biodisponibilidad oral de la secuencia HLPLP en SHRs, demostrándose una rápida absorción tras su administración oral, y también su rápida biotransformación, por acción de las peptidasa plasmáticas, en los fragmentos LPLP y HLPL que se distribuyen a través de la circulación sanguínea (Sánchez-Rivera et al., 2014). Además, tanto VPP, IPP como HLPLP han sido detectados en plasma humano tras su administración oral (van Platerink et al., 2006; Foltz et al., 2007).

2.6 Mecanismos de acción de péptidos antihipertensivos

La intervención de mecanismos de acción antihipertensivos además de o diferentes a la inhibición de la ECA, también puede explicar la falta de correlación entre los efectos *in vitro* e *in vivo* de los péptidos antihipertensivos. Además de la escasa biodisponibilidad de los péptidos

antihipertensivos, también se cuestiona el hecho de que puedan actuar *in vivo* sólo a través de la inhibición de la ECA. Incluso, algunos autores especularon sobre la posibilidad de que determinados péptidos antihipertensivos no necesitaran ser absorbidos ya que ejercerían su acción a través de receptores del tracto gastrointestinal (Yamada et al., 2002).

En los últimos años distintos estudios han evidenciado la posibilidad de que los péptidos antihipertensivos puedan actuar sobre componentes del SRAA diferentes de la ECA, así como a través de cualquier otro mecanismo de regulación de la PA (Udenigwe y Mohan, 2014).

En cuanto al SRAA, diferentes hidrolizados de proteínas alimentarias y péptidos poseen actividad inhibidora *in vitro* de la renina (Udenigwe et al., 2009; Fitzgerald et al., 2012; Udenigwe y Aluko, 2012a; Udenigwe et al., 2012b; Ajibola et al., 2013; Onuh et al., 2013; He et al., 2013a), y algunos de ellos también efecto antihipertensivo en SHRs, mostrando disminuciones de la PAS de 12-30 mm Hg (Girgih et al., 2011; Li et al., 2011; He, et al., 2013a; He et al., 2013b). Sin embargo, los estudios que demuestran la inhibición *in vivo* de la renina son escasos: por ejemplo, tras la administración crónica del pentapéptido RVPSL derivado de huevo a SHRs, se observó una represión de la expresión del gen que codifica la renina, aunque también de los genes que codifican la ECA y el receptor AT_1 (Yu et al., 2014).

El receptor AT_1 también es la diana de algunos de los péptidos antihipertensivos derivados de LF que fueron caracterizados por nuestro grupo en ensayos funcionales *ex vivo* (Fernández-Musoles et al., 2014). En la Tabla 4, se resume la inhibición de la vasoconstricción inducida por Ang II.

Tabla 4. Efectos inhibitorios de péptidos derivados de LF sobre la vasoconstricción inducida por Ang II (adaptada de Manzanares et al., 2015)

Péptido	Inhibición de la vasoconstricción inducida por Ang II (%)[1]
RRWQWR (20 μM)	21
LIWKL (20 μM)	30
RPYL (20 μM)	44
RPYL (200 μM)	80
Pepsina LFH (100 μg/mL)	25

[1] Expresado como porcentaje respecto al control

Como se puede observar, tanto el hidrolizado de LF como los péptidos individuales inhiben la vasoconstricción inducida por Ang II, destacando la secuencia RPYL. Por tanto, el bloqueo de los receptores AT_1 podría ser otro mecanismo de acción que, junto con la inhibición de la ECA, contribuyan al efecto antihipertensivo de los péptidos derivados de LF (Fernández-Musoles et al., 2014).

Por otro lado, los péptidos antihipertensivos no sólo pueden actuar sobre el SRAA, sino que también pueden ejercer su efecto a través de cualquier otro mecanismo regulador de la PA. En este contexto, los inhibidores de determinados componentes del sistema ET, como los inhibidores de la ECE y los antagonistas de los receptores de ET, están adquiriendo relevancia en los últimos años como tratamiento antihipertensivo (Kędzierski y Yanagisawa, 2001). Este es el caso de algunos péptidos e hidrolizados derivados de LF, capaces de inhibir *in vitro* la ECE y también la vasoconstricción ECE-dependiente (Fernández-Musoles et al., 2010; Fernández-Musoles et al., 2013a). Aunque la inhibición *in vivo* de la ECE por los péptidos antihipertensivos no ha sido demostrada, este mecanismo podría sumarse al de la inhibición de la ECA en el caso del hidrolizado de LF obtenido con pepsina. También se ha descrito la inhibición de la formación de ET-1 en células endoteliales

tratadas con el péptido de secuencia ALPMIHR derivado de β-Lg (Maes et al., 2004).

Asimismo, también se ha señalado la vasodilatación mediada por NO como otro posible mecanismo de acción de los péptidos antihipertensivos. Se ha sugerido que el NO es el responsable de los efectos vasorrelajantes dependientes de endotelio observados en arterias mesentéricas de SHRs tratadas con los péptidos α-lactorfina (YGLF) y β-lactorfina (YLLF), provenientes de la α-La y β-Lg respectivamente (Nurminen et al., 2000; Sipola et al., 2002). Este mismo efecto a nivel vascular también se describió para diferentes secuencias antihipertensivas derivadas de huevo (Garcia-Redondo et al., 2010).

La eNOS, enzima clave en la producción del NO en el endotelio a partir de L-arginina, también es diana molecular de los péptidos antihipertensivos. Se ha descrito su incremento tanto a nivel de mRNA como de proteína en aorta de SHRs tratadas con VPP e IPP (Yamaguchi et al., 2009) o con un hidrolizado de caseína (Sánchez et al., 2011). También se ha señalado un incremento en la fosforilación de la eNOS en células endoteliales tratadas con péptidos inhibidores de la ECA (Shimizu et al., 2010; Ko et al., 2012). Asimismo, se ha relacionado el efecto antihipertensivo de péptidos ricos en Arg con un aumento del aminoácido en el endotelio y por tanto con una mayor producción de NO (Udenigwe et al., 2012c; Doyen et al., 2014). Estos péptidos también mostraron una moderada inhibición *in vitro* de la ECA y de la renina, lo cual sugiere que diferentes mecanismos podrían estar implicados en sus efectos antihipertensivos.

Las técnicas ómicas ofrecen una visión global para el abordaje de las bases moleculares de los péptidos antihipertensivos, representando una estrategia muy útil para elucidar los diferentes mecanismos de acción sobre los que actúan estos péptidos. Sin embargo, hasta la fecha son

pocos los estudios que emplean esta estrategia. Cabe destacar el análisis de expresión génica, empleando micromatrices de DNA, llevado a cabo en aorta de SHRs tras la administración crónica de VPP e IPP (Yamaguchi et al., 2009). En general, los cambios en la expresión génica fueron pocos y de escasa magnitud. Se observó un incremento moderado en la expresión del gen que codifica la eNOS y el gen que codifica la conexina 40, proteína estructural de las uniones intercelulares. Relacionado con el sistema del ácido araquidónico, el gen que codifica la ciclooxigenasa 1 (COX-1) mostró un ligero aumento en la expresión, así como también se observó un descenso en la expresión de la subunidad B del factor nuclear kappa (relacionado con función vascular) y del receptor activador de la proliferación de peroxisomas (PPARγ). Es de destacar que no hubo cambios significativos en la expresión de genes asociados con el SRAA o con el sistema de coagulación de la sangre (Yamaguchi et al., 2009). Recientemente, se utilizó la misma tecnología para determinar el mecanismo molecular del efecto antihipertensivo de una leche fermentada con *Lb. helveticus.* Se encontró que los genes cuya expresión se incrementó significativamente estaban relacionados con la síntesis de NO, la proliferación celular, ET, y la trombolisis (Chen et al., 2015).

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Objetivos

La prevención y/o tratamiento de la hipertension a base de una dieta diaria que aporte péptidos antihipertensivos es una opción atractiva frente a la utilización de fármacos que pueden producir efectos secundarios indeseables. En este contexto, las proteínas de la leche, principalmente la caseína (CN), son una de las fuentes principales de péptidos antihipertensivos y la base de los productos comerciales con efecto antihipertensor. La fermentación microbiana es, junto con la hidrólisis enzimática, uno de los procesos más utilizados para generar péptidos antihipertensivos. Hasta la fecha, este proceso se ha centrado principalmente en la utilización de bacterias lácticas (BAL), mientras que otros grupos de microorganismos, como las levaduras no convencionales con reconocido potencial proteolítico, han sido menos explorados.

La lactoferrina (LF), proteína componente del suero lácteo, tiene múltiples funciones biológicas y nuestro grupo de trabajo demostró que diferentes hidrolizados enzimáticos de LF y también péptidos derivados presentaban capacidad antihipertensiva en SHRs tras su administración oral, y por tanto un uso potencial como ingrediente alimentario funcional. Sin embargo, el mecanismo de acción de estos péptidos no se ha establecido completamente de manera fehaciente.

Teniendo en cuenta estos antecedentes, esta Tesis Doctoral tiene dos objetivos:

Objetivo I. Explorar el potencial de las levaduras no convencionales para generar hidrolizados y péptidos antihipertensivos derivados de CN y LF.

Objetivo II. Profundizar en el mecanismo de acción involucrado en el efecto antihipertensivo de los péptidos derivados de LF, incluyendo aquellos generados previamente mediante hidrólisis enzimática y también las nuevas secuencias generadas por las levaduras no convencionales.

Estos objetivos se abordan en los siguientes capítulos que se corresponden con sendos artículos científicos publicados:

Objetivo I.

Capítulo I. Obtención de hidrolizados antihipertensivos derivados de CN y LF utilizando levaduras no convencionales.

Se emplearán cepas de levaduras pertenecientes a las especies GRAS *Debaryomyces hansenii*, *Kluyveromyces lactis* y *Kluyveromyces marxianus,* se evaluará la capacidad de los hidrolizados generados para inhibir *in vitro* la actividad de la enzima conversora de angiotensina (ECA), y se estudiará su efecto antihipertensivo en ratas espontáneamente hipertensas (SHRs). Se seleccionará el hidrolizado más potente para la posterior identificación de los péptidos responsables del efecto antihipertensivo.

Capítulo II. Evaluación de la capacidad de las levaduras no convencionales para generar secuencias derivadas de CN con efecto antihipertensivo demostrado.

Se emplearán las cepas de *D. hansenii*, *K. lactis* y *K. marxianus* para generar hidrolizados de CN y se comprobará mediante espectrometría de masas en tándem (HPLC-MS/MS) la generación de las secuencias peptídicas IPP, VPP, RYLGY, AYFYPEL, LHLPLP y HLPLP con reconocida capacidad antihipertensiva en modelos animales preclínicos y/o ensayos clínicos con pacientes.

Objetivo II.

Capítulo III. Identificación de los péptidos derivados de LF generados por levaduras no convencionales y caracterización de su efecto antihipertensivo.

Se identificarán mediante HPLC-MS/MS los péptidos más abundantes presentes en el hidrolizado de LF seleccionado en el capítulo I, y se elegirán aquellos que por su abundancia o secuencia pudieran ser responsables de su efecto antihipertensivo. La caracterización de las secuencias incluirá la capacidad *in vitro* de inhibición de la ECA, su efecto antihipertensivo y la inhibición *in vivo* de la ECA en SHRs, así como su estabilidad gastrointestinal.

Capítulo IV. Estudio de la relevancia del sistema renina-angiotensina-aldosterona (SRAA) en el efecto antihipertensivo de péptidos derivados de LF.

Se utilizará un modelo de hipertensión inducida en ratas Wistar-Kyoto mediante la infusión continua de angiotensina I (Ang I) o angiotensina II (Ang II) para comprobar los efectos de los péptidos sobre la hipertensión dependiente de la ECA o de la activación de los receptores AT_1, respectivamente.

Capítulo V. Estudio de la relevancia de otros sistemas de regulación de la presión arterial (PA) diferentes del SRAA en el efecto antihipertensivo de los péptidos derivados de LF.

Se utilizá como modelo células endoteliales humanas para evaluar el efecto de los péptidos derivados de LF sobre la expresión génica. Dicho efecto se estudiará mediante matrices de PCR cuantitativa dirigidas a genes de vías de señalización relacionadas con la regulación de la PA.

Resultados

Objetivo I

Capítulo I. Dairy yeasts produce milk protein-derived antihypertensive hydrolysates. *Food Research International* 53: 203−208 (2013).

Capítulo II. Dairy *Debaryomyces hansenii* strains produce the antihypertensive casein-derived peptides LHLPLP and HLPLP. *LWT - Food Science and Technology* 61: 550-556 (2015).

Dairy yeasts produce milk protein-derived antihypertensive hydrolysates

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Food Research International 53:

203−208 (2013)

ABSTRACT

The potential of 20 dairy yeast strains belonging to *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Kluyveromyces marxianus* species was examined for the production of milk protein-derived antihypertensive hydrolysates. For this purpose yeast strains were grown in microbiological medium with casein or lactoferrin as sole nitrogen source, and the inhibitory effects of casein and lactoferrin hydrolysates (CSHs and LFHs) on angiotensin I-converting enzyme (ACE) activity were determined. Based on the ACE-inhibitory activity, four CSHs and five LFHs were selected, and permeate fractions with molecular masses lower than 3 kDa (pCSHs and pLFHs) were obtained. *In vitro* ACE-inhibitory potencies (IC_{50}) of permeates varied from 18.8 to 87.6 μg/ml (pCSHs) and from 50.2 to 500 μg/ml (pLFHs). *K. marxianus* Km2 strain grown on either casein or lactoferrin produced the most potent permeates. pCSHs and pLFHs were orally administered to spontaneously hypertensive rats (SHRs) and exerted *in vivo* antihypertensive effect. In conclusion, the present study contributes to a better insight into bioactive compounds produced by dairy yeasts and shows the feasibility of selected yeasts to produce orally effective antihypertensive milk protein-derived hydrolysates.

Keywords: *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, casein-derived peptides, lactoferrin-derived peptides, ACE inhibition.

1. Introduction

Food derived bioactive peptides are attracting increasing interest because of their variety and multifunctionality. Undoubtedly, those with blood pressure-lowering effects are receiving increasing attention due to the worldwide growing prevalence of hypertension (Kearney et al., 2005). One of the main targets for the treatment of hypertension is the renin angiotensin system (RAS), and its inhibition at three possible levels, angiotensin-converting enzyme (ACE), upstream renin activity or downstream angiotensin receptors, is the pharmacological basis for commonly used antihypertensive drugs (Fragasso et al., 2012). ACE, which hydrolyzes both the inactive angiotensin I into vasoconstrictor angiotensin II and the vasodilator bradykinin into an inactive metabolite, is also the main target for antihypertensive food-derived peptides developed as an alternative to drugs (reviewed in Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012).

Nowadays milk proteins are the main source of antihypertensive peptides. Among the approaches for releasing ACE-inhibitory peptides from intact milk proteins, fermentation with proteolytic lactic acid bacteria (LAB) to partially digest the caseins during the manufacture of dairy products is a successful strategy (reviewed in Hernández-Ledesma, Contreras, & Recio, 2011). Moreover, milk fermented with *Lactobacillus helveticus,* containing the casein-derived peptides VPP and IPP, has shown significant antihypertensive effects in rats and humans (Hata et al., 1996; Nakamura, Yamamoto, Sakai, & Takano, 1995; Seppo, Jauhiainen, Poussa, & Korpela, 2003; Seppo, Kerojoki, Suomalainen, & Korpela, 2002). Also enterococci from dairy origin are able to hydrolyse casein into peptides with ACE-inhibitory activity and antihypertensive effect (Muguerza et al., 2006; Chaves-López et al., 2011); however the pathogenic potential of some

Enterococcus strains (Franz, Holzapfel, & Stiles, 1999) may hamper their use in food production.

Yeast products have been used for many years as ingredients and additives in food processing, although their potential bioactivity has been less investigated (Dawson, 2002; Abbas, 2006). Yeasts isolated from dairy environments have proteolytic character (Jakobsen & Narvhus, 1996) and thus potential for releasing bioactive peptides. *Kluyveromyces marxianus* was pointed out as a promise candidate to generate antihypertensive peptides from the whey proteins α-lactalbumin and β-lactoglobulin, alone (Belem, Gibbs, & Lee, 1999) or in combination with *Lactobacillus rhamnosus* (Hamme, Sannier, Piot, & Bordenave-Juchereau, 2009a; Hamme, Sannier, Piot, Didelot, & Bordenave-Juchereau, 2009b). Recently *K. marxianus* isolated from Colombian kumis, a traditional low alcoholic fermented cow milk, was able to produce fermented milk with ACE-inhibitory activity (Chaves-López et al., 2012). However the *in vivo* antihypertensive effect of casein- and whey-derived bioactive peptides generated by yeast strains has not been demonstrated yet.

In contrast to the aforementioned milk proteins, the potential of bovine lactoferrin, a well-characterized component of milk whey, as a source of antihypertensive peptides has been much less explored. We have reported the inhibitory effects of enzymatic lactoferrin hydrolysates on ACE activity and also their antihypertensive effect in spontaneously hypertensive rats (SHRs), suggesting their potential application as nutraceutical in the treatment of hypertension (Fernández-Musoles et al., 2013; Ruiz-Giménez et al., 2012). However, there is a lack of information about the feasibility of using proteolytic microorganisms to generate new lactoferrin-derived peptides with antihypertensive effects.

The objective of the present study was to further investigate the potential of yeasts to generate milk protein-derived peptides with

antihypertensive effect. For this purpose, different strains of *Debaryomyces hansenii*, *Kluyveromyces lactis* and *K. marxianus* isolated from cheeses were screened for their ability to grow in media with casein or lactoferrin as sole nitrogen source and to produce protein hydrolysates containing ACE-inhibitory peptides. Selected casein and lactoferrin raw hydrolysates (CSHs and LFHs, respectively) were ultrafiltered to obtain permeates enriched in peptides of molecular weight lower than 3 kDa (pCSHs and pLFHs) which ACE-inhibitory potency was evaluated. Finally the antihypertensive effect of most potent permeates was assessed in SHRs.

2. Materials and methods

2.1 Materials

Bovine lactoferrin was provided by FrieslandCampina Domo (Zwolle, The Netherlands). Casein (Promilk 85) was obtained from Ingredia (Arras Cedex, France). ACE from porcine kidney, captopril, and bicinchoninic acid protein assay kit were purchased from Sigma (St. Louis, MO, USA). Glucose was obtained from Panreac (Barcelona, Spain), bacteriological peptone was purchased from Cultimed (Barcelona, Spain) and yeast extract and agar were acquired from Pronadisa (Madrid, Spain). o-aminobenzoylglycyl-p-nitrophenylalanylproline was provided by Bachem Feinchemikalien (Bubendorf, Switzerland)

2.2 Yeast strains and growth conditions

Twenty yeast strains belonging to *D. hansenii* (10), *K. lactis* (8) and *K. marxianus* (2) species isolated from artisanal ewes' and goats' milk cheeses were used in this study. Yeast strains were maintained on GPYA medium (2 % glucose, 0.5 % yeast extract, 0.5 % peptone and 2 % agar, pH 5.5).

2.3 Preparation of lactoferrin and casein hydrolysates

Stock solutions of lactoferrin and casein were sterilized by autoclaving at 121°C, 15 min. Lactoferrin (1.5 % lactoferrin, 2 % glucose) and casein medium (2 % casein, 2 % glucose) were inoculated with 10^8 and 10^6 cells/ml, respectively, from pre-cultured strains on GPY (GPYA without agar), incubated at 28°C and 100 rpm in an orbital shaker. For the initial screening, yeast strains were grown in 4 ml of casein or lactoferrin medium. Selected strains were cultured in five 200 ml-batches of casein

and lactoferrin medium. At the end of the incubation period (7 days for casein medium and 14 days for lactoferrin medium), yeast cells were eliminated by centrifugation (13000 rpm, 10 minutes), and the supernatants were considered as casein and lactoferrin hydrolysates (CSHs and LFHs). Proteolysis and ACE-inhibitory activity of hydrolysates were determined as specified further.

Selected CSHs and LFHs were subjected to ultrafiltration through a VivaFlow 50 crossflow cassette with a cut-off polyethersulfone membrane (Vivascience, Sartorius Stedim Biotech, Aubagne, France). Resulting permeates, enriched in peptides of molecular weight lower than 3 kDa were named pCSHs and pLFHs. Protein concentration of permeates was estimated by the bicinchoninic acid method (BCA) using bovine serum albumin as standard (Ruiz-Giménez et al., 2012).

2.4 Determination of extent of proteolysis

CSHs and LFHs were analysed by reversed phase-high performance liquid chromatography (RP-HPLC) using a Waters system (Waters Corporation, Milford, MA) equipped with a 1525 Binary HPLC pump, a 2996 Photodiode Array Detector and a 717 plus Autosampler. Hydrolysates (90 µl) were applied to a Symmetry® C_{18} 5 µm, 4.6 $\times$ 150 mm column (Waters). The column was developed at a flow rate of 1 ml/min at 40°C. Elution was performed with a linear gradient of solvent B (acetonitrile with 1% TFA) in solvent A (water with 1% TFA) from 0 to 80% in 60 min. Detection of peptides and proteins was carried out at 214 nm. Extent of proteolysis, expressed as percentage, was calculated from the chromatographic peak areas of either casein or lactoferrin in hydrolysates at the end of the incubation time versus peak areas at time zero.

2.5 *In vitro* assay of ACE-inhibitory activity

In vitro ACE-inhibitory activity of CSHs and LFHs 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 by the action of ACE.

Effects on ACE activity of hydrolysates (20 µl) were expressed as percentage of ACE activity inhibition calculated with respect to a control without hydrolysate. Duplicate assays were done and ACE-inhibitory activity expressed as mean percentage ± SEM.

The IC_{50} value of pCSHs and pLFHs was defined as the protein 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 10.0 (SPSS Inc., Chicago, IL, USA).

2.6 *In vivo* assay of antihypertensive effect in SHRs

Ten male SHRs weighing 250–300 g were used (Charles River Laboratories, Barcelona, Spain). 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 to the Directives of the European Community on this subject. The study was approved by the 'Ethics Committee for Animal Welfare' of 'La Fe' Hospital.

Indirect measurement of systolic blood pressure (SBP) in awake restrained rats was carried out by the non-invasive tail-cuff method using computer-assisted Non-Invasive Blood Pressure equipment (LE5001 unit with LE5160R cuff & transducer, Panlab Harvard Apparatus, Cornellá,

Barcelona, Spain). Permeates (200 mg/kg) were orally administered by gastric intubation in 750 µl of physiological saline. Before the measurements, rats were kept at 37°C during 15 min to make the pulsations of the tail artery detectable. The SBP was measured before peptide intake (zero time), 1, 2, 3, 4 and 24 h after intake. Physiological saline (750 µl) and captopril (50 mg/kg) served as negative and positive controls, respectively. Each value of SBP was obtained by averaging at least three consecutive and successful measurements without disturbance of the signal. Changes in SBP were calculated as the absolute difference (in mm Hg) with respect to the basal values of measurements obtained just before starting the treatments.

3. Results

3.1 Selection of ACE-inhibitory casein and lactoferrin hydrolysates

All yeast strains tested were able to grow on microbiological medium containing either casein or lactoferrin as sole nitrogen source. Analysis of hydrolysates by RP-HPLC showed that, as a general trend, yeasts completely hydrolysed casein (approximately 90% of the initial concentration) after 7 days of incubation. By contrast, approximately 50 % of the initial lactoferrin concentration was hydrolysed after 14 days of growth. Representative recordings of peptide profiles can be seen in Fig. 1. Therefore we obtained forty different protein hydrolysates which *in vitro* ACE-inhibitory effects are reported in Table 1. As can be seen CSHs and LFHs produced ACE inhibition. *D. hansenii* strains produced CSHs with ACE inhibition ranging from 20 to 79 %, similar to that provoked by *D. hansenii* LFHs (27 to 77 %). With respect to *Kluyveromyces* yeasts, the highest ACE inhibition provoked by casein-derived peptides was about 55%, corresponding to hydrolysates generated by *K. lactis* Kl3 and *K. marxianus* Km2. With the exception of LFH generated by *K. lactis* Kl7 which failed to inhibit ACE, lactoferrin-derived peptides exhibited higher ACE inhibition (50-77 %) than that found for peptides derived from casein (18-54%).

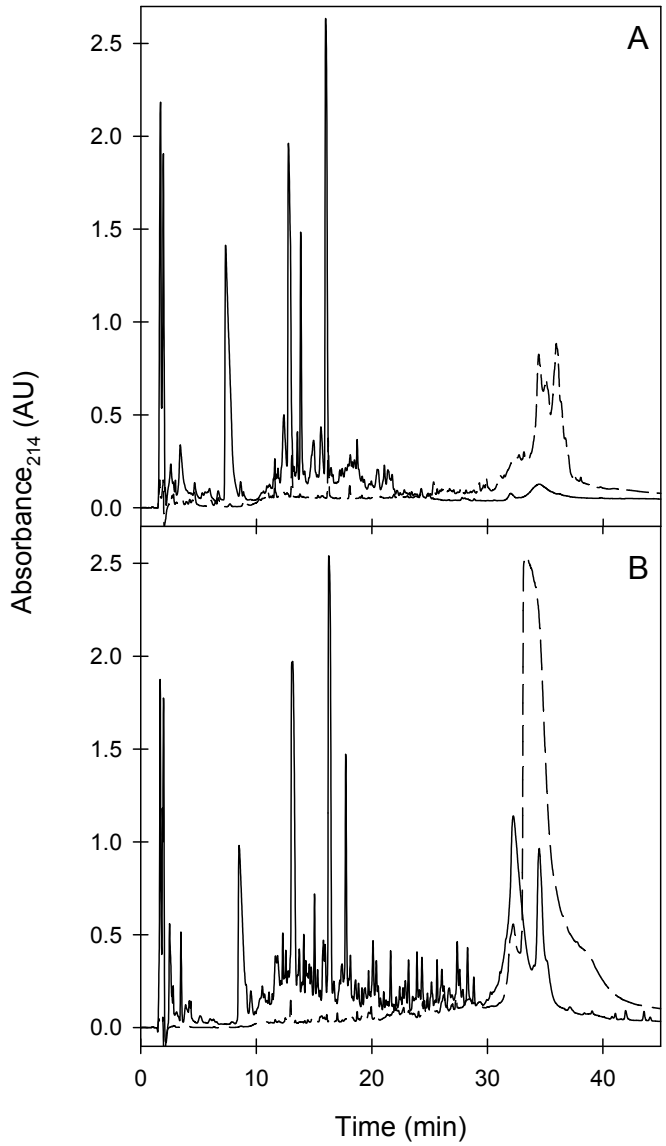

Figure 1. RP-HPLC chromatograms of casein (CSH; panel A) and lactoferrin (LFH; panel B) hydrolysates produced by *D. hansenii* Dh4. Dashed lines represent initial casein and lactoferrin concentrations.

Table 1. ACE-inhibitory activity of casein (CSH) and lactoferrin (LFH) hydrolysates produced by yeast strains.

		ACE-inhibitory activity[b] (%)	
Species	Strain[a]	CSH	LFH
D. hansenii	Dh1	39	40
	Dh2	36	40
	Dh3	37	33
	Dh4	65	77
	Dh5	45	27
	Dh6	27	76
	Dh7	37	47
	Dh8	79	62
	Dh9	20	53
	Dh10	22	54
K. lactis	Kl1	18	61
	Kl2	47	57
	Kl3	52	52
	Kl4	39	48
	Kl5	49	62
	Kl6	39	69
	Kl7	32	n.d.
	Kl8	44	77
K. marxianus	Km1	48	54
	Km2	54	73

n.d. not detected
[a]All yeast strains were isolated from Spanish cheeses.
[b]ACE-inhibitory activity determined using 20 µl of yeast hydrolysates. Mean values for two replicas.

On the basis of these results, 9 hydrolysates (CSHs produced by strains Dh4, Dh8, Kl3 and Km2 and LFHs generated by Dh4, Dh6, Kl6, Kl8 and Km2) were selected for further studies.

3.2 ACE-inhibitory potencies of selected casein and lactoferrin hydrolysates

Ultrafiltration of crude hydrolysates to obtain fractions enriched in low molecular weight peptides is a common strategy used to increase *in vitro* ACE-inhibitory potency and potential antihypertensive effect. Thus selected hydrolysates, were subjected to ultrafiltration through a 3 kDa cut-off membrane and further concentration-response curves of the resulting permeates (pCSHs and pLFHs) allowed the determination of their IC_{50} values as summarized in Table 2. In general the higher inhibitory potency as indicated by lower IC_{50} values corresponded to pCSHs. With the exception of Dh8 pCSH, all of them exhibited IC_{50} values lower than 50 µg/ml. By contrast ACE-inhibitory potency of pLFHs varied over a 10-fold range. Interestingly results pointed out that *K. marxianus* Km2 strain, which permeates obtained either from casein or from lactoferrin showed the highest inhibitory potency. Fig. 2 shows representative recordings of concentration-response curves.

Table 2. Inhibitory potency[a] of casein (pCSH) and lactoferrin (pLFH) permeates on ACE activity.

	IC_{50} (µg/ml)	
Species	pCSH	pLFH
D. hansenii Dh4	27.7 ± 1.0	89.6 ± 3.7
D. hansenii Dh6	n.d.	460.3 ± 23.4
D. hansenii Dh8	87.6 ± 3.3	n.d.
K. lactis Kl3	39.1 ± 2.9	n.d.
K. lactis Kl6	n.d.	140.2 ± 9.2
K. lactis Kl8	n.d.	500 ± 32.3
K. marxianus Km2	18.8 ± 1.3	50.2 ± 2.7

n.d. not determined
[a]Inhibitory potency is expressed as IC_{50} and is the mean ± SEM of at least three independent experiments.

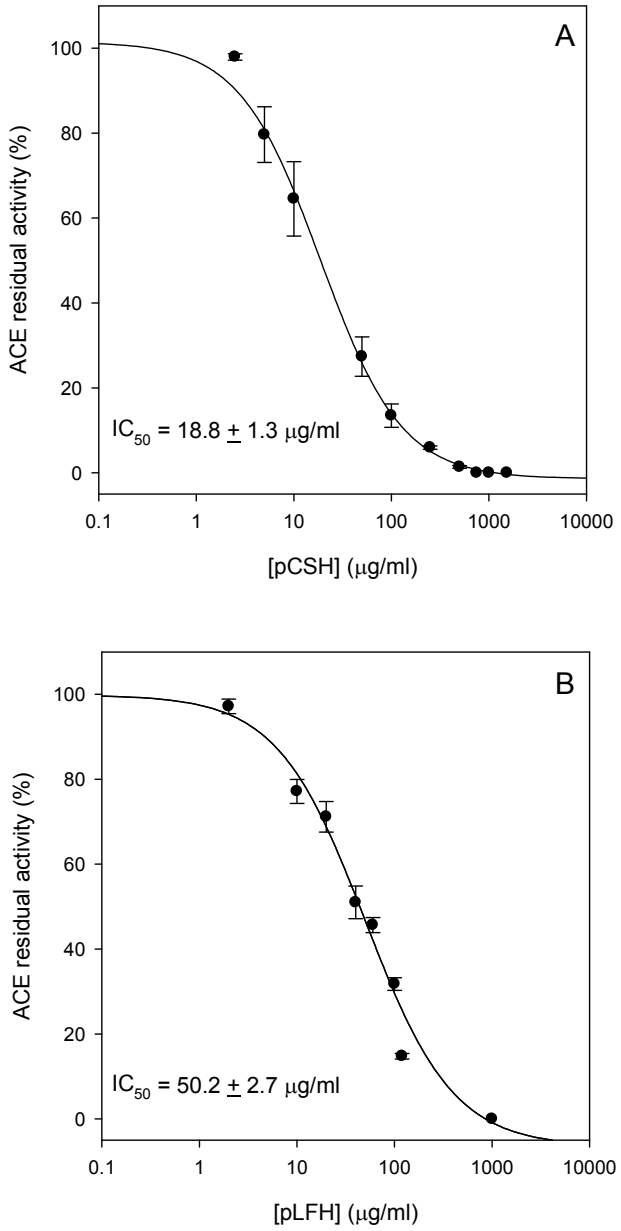

Figure 2. Effect of the concentration of casein (pCSH; panel A) and lactoferrin (pLFH; panel B) permeates produced by *K. marxianus* Km2 on ACE residual activity. IC_{50} is the mean ± SEM of at least three independent experiments.

Based on these results, 6 permeates (pCSHs produced by strains Dh4, Kl3 and Km2 and pLFHs generated by Dh4, Kl6 and Km2) were selected for further studies of their *in vivo* antihypertensive effect

3.3 Antihypertensive effects of casein and lactoferrin permeates

The average SBP for all measurements carried out in SHRs from the eight experimental groups before treatment intake (zero time) was 212 ± 1 mm Hg (n = 63). Oral administration of selected permeates at a dose of 200 mg/kg induced significant reductions in SBP as shown in Fig. 3, together with the lack of effect of oral saline and the antihypertensive effect of captopril (50 mg/kg) for comparison. With respect to pCSHs (panel A), the effects produced by Km2 and Dh4 strains were very similar. The decrease of SBP was maximal 2 h after the oral administration (−22 ± 6 mm Hg and −21 ± 2 mm Hg, respectively), and it remained significant up to 4 h post-administration. By contrast oral administration of Kl3 pCSH led to a slight decrease in SBP, which it was significant only at 4 h post-administration (−9 ± 2 mm Hg). According to the time course shown in panel B, the three pLFHs caused significant antihypertensive effects when compared to the control group. The decrease of SBP was maximal at 1 h post-administration for Km2 (−24 ± 1 mm Hg) and Dh4 (−18 ± 2 mm Hg) permeates. The effect of Km2 pLFH remained significant up to 4 h postadministration whereas the SBP decreased caused by Dh4 pLFH reverted at that time point. Although the effect of Kl6 permeate was lower than those described for the other two pLFHs, it remained significant and practically constant up to 4 h.

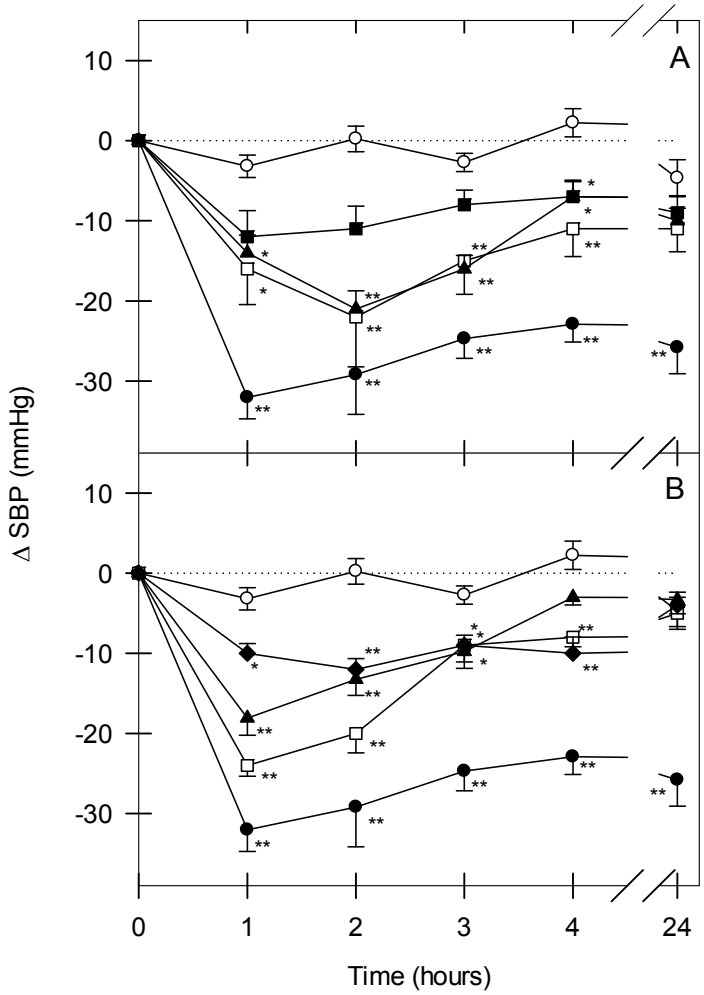

Figure 3. Time course of systolic blood pressure changes (ΔSBP) after oral administration of physiological saline (○), captopril (●, 50 mg/kg) and permeates (200 mg/kg) to SHR. (A) Effect of casein permeates (pCSHs): (▲), *D. hansenii* Dh4 pCSH; (■), *K. lactis* Kl3 pCSH and (□) *K. marxianus* Km2 pCSH. (B) Effect of lactoferrin permeates (pLFHs): (▲), *D. hansenii* Dh4 pLFH; (◆), *K. lactis* Kl6 pLFH and (□) *K. marxianus* Km2 pLFH. Pressure changes from baseline are expressed in absolute values (mm Hg) and data are mean ± SEM from at least 4 determinations, **P*<0.05 versus control group, ***P*<0.01 versus control group (one-way ANOVA followed by Dunnett multiple comparison tests).

4. Discussion

Generation of bioactive peptides from milk proteins requires their release by either enzymes or proteolytic microorganisms. The production of antihypertensive peptides by LAB milk fermentation is well recognized. By contrast few studies exploit the proteolytic potential of yeasts. *D. hansenii*, *K. lactis* and *K. marxianus*, frequently found associated with milk, cheese and other dairy products (Belloch, Querol, & Barrio, 2011; Büchl & Seiler, 2011), are considered GRAS (Generally Recognized As Safe) microorganisms, and their contribution to proteolysis during cheese ripening is well established (Sousa, Ardö, & McSweeney, 2001). In this work, we used 20 proteolytic yeast strains to produce milk protein-derived hydrolysates containing peptides with ACE-inhibitory and antihypertensive effects. Casein, as the major milk protein and, nowadays, the main source of antihypertensive peptides, and lactoferrin, as a minor component of milk whey with potential to generate new antihypertensive sequences, were included in the study.

Yeast strains tested were able to produce ACE-inhibitory hydrolysates during growth in casein or lactoferrin as sole nitrogen source. In the conditions tested, yeasts efficiently degraded casein since almost complete hydrolysis of the protein was observed. By contrast only half hydrolysis of the initial lactoferrin was achieved, probably due to the tertiary structure of the protein which makes it relatively resistant to proteolysis (Lönnerdal & Iyer, 1995). Regarding casein degradation by LAB, it is well established that firstly a cell envelope-associated proteinase acts on casein to provide short peptides which became substrates for a range of intracellular proteinases and peptidases (Kunji, Mierau, Hagting, Poolman, & Konings, 1996). With respect to yeasts, caseinolytic activity is well documented but few reports deal with the characterization of the proteolytic system. Kumura et al., (2002) reported that a cell-wall associated protease

of *D. hansenii* acted on β-casein whereas α_S-casein degradation was attributed to an intracellular protease liberated by cell lysis. By contrast, extracellular proteolytic activity on α_S- and β-casein for *Y. lipolytica* and *D. hansenii* strains isolated from cheese has been described (Gardini et al., 2006). Recently a novel extracellular protease from *K. marxianus* has been purified and characterized as serine protease although hydrolytic activity against casein was not evaluated (Foukis, Stergiou, Theodorou, Papagianni, & Papamichael, 2012). Hydrolysis of milk whey proteins such as β-lactoglobulin and α-lactalbumin by *K. marxianus* proteases has been discussed (Belem et al., 1999; Hamme, Sannier, Piot, Didelot, et al., 2009) but there are no reports about lactoferrin degradation by yeasts.

ACE-inhibitory potencies similar to those described here for yeast pCSHs (IC_{50} values from 18.8 to 27.7 μg/ml) were reported for fermented milk produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* or *Lactococcus. lactis* subsp *cremoris* (IC_{50} values from 8 to 11.2 μg/ml; (Gobetti, Ferranti, Smacchi, Gofrredi, & Addeo, 2000), by *Enteroccocus faecalis* strains (IC_{50} values between 34 and 59 μg/ml; Muguerza et al., 2006) and for casein hydrolysates produced with proteinase from *L. helveticus* CP790 (IC_{50} values ranging from 11 to 24 μg/ml; Yamamoto, Akino, & Takano, 1994). ACE-inhibitory potency of yeast fermented milks after simulated gastrointestinal digestion ranged from 10.37 to 29.10 μg/ml (Chaves-López et al., 2012). Among them, *K. marxianus* fermented milk exhibited the highest inhibitory potency in agreement with our results and, remarkably, peptides released did not confer bitter taste to milk. With respect to yeast pLFHs, their ACE inhibition was lower than the previously reported value of enzymatic LFHs (IC_{50} = 1.3-14.3 μg/mL; Ruiz-Giménez et al., 2012; Fernández-Musoles et al., 2013).

The *in vivo* experiments showed a clear antihypertensive effect for all permeates tested. Nevertheless, the effect of yeast pCSHs and pLFHs

on SBP was transient and less pronounced than the effect produced by administration of captopril, the gold standard drug for ACE inhibition. However, it is not the objective of this study to replace ACE-inhibitory drugs but develop functional ingredients that integrated in food would provide a preventive more than curative treatment for hypertension without possible side effects.

Blood pressure-lowering effects in SHRs have been described for LAB-fermented products. Maximum decrease of SBP values similar to those described here for Km2 and Dh4 pCSHs were described for fermented milk samples (21.8 ± 4.2 mm Hg), which hypotensive properties are related to the formation of the tripeptides VPP and IPP (Nakamura et al., 1995; Yamamoto, Maeno, & Takano, 1999;). Higher antihypertensive effects were observed when *E. faecalis* fermented milk samples were orally administered to SHRs (34.81 ± 4.48 mm Hg; Muguerza et al., 2006), although the effects were mainly attributed to the casein-derived hexapeptide HLPLP (Quirós et al., 2007). Moreover, over the last two decades, a large number of casein-derived peptides obtained by fermentation and enzymatic hydrolysis with proven antihypertensive effects in SHRs (SBP decreases from -2 to -34 mm Hg) have been identified (Martínez-Maqueda et al., 2012).

Yeast pLFHs showed a significant reduction of SBP ranging from -12 to -24 mm Hg. Permeate produced by Km2 strain exerted the highest antihypertensive effect on SHRs in our study, slightly higher than that described for pepsin (-15.4 ± 3.8 mm Hg; Ruiz-Giménez et al., 2012) and proteinase K (-19 ± 7 mm Hg; Fernández-Musoles et al., 2013) permeates. Contrary to casein-derived peptides, so far only five LF-derived peptides of sequences RRWQWR, WQ (Ruiz-Giménez et al., 2010), RPYL, LIWKL and LNNSRAP (Ruiz-Giménez et al., 2012) have shown antihypertensive effects after oral administration to SHRs. Among them, the most potent

peptide, LIWKL, provoked a SBP reduction (-25.3 ± 3.5 mm Hg) similar to that found here for Km2 pLFH. To what extent already described casein- and lactoferrin-derived antihypertensive peptides are responsible for the observed ACE inhibiting and blood pressure-lowering effects of yeast pCSHs and pLFHs requires further characterization studies.

Our results show the possibility of using GRAS dairy yeasts as producers of bioactive peptides. To the best of our knowledge this is the first report showing the antihypertensive effects of milk protein-derived hydrolysates generated by yeasts. Future efforts will be directed to identify the active peptide sequences and characterise the mode of action of the proteolytic system of the selected strains. Furthermore the ability of *K. marxianus* and *K. lactis* to ferment lactose would allow the fermentation approach to develop new milk-based products with antihypertensive effects.

5. Acknowledgements

This work was supported by grant AGL2010-21009 from 'Ministerio de Educación y Ciencia - FEDER', Consolider Ingenio 2010, Fun-C-Food, CSD2007-00063, RETICS RD06/0026/0006 from 'Instituto de Salud Carlos III' and ACOMP/2012/011 from 'Generalitat Valenciana'. A. García-Tejedor and B. Padilla are recipients of predoctoral fellowships from 'Ministerio de Educación y Ciencia' (BES-2011-044424) and CSIC (JAE-PreDoc), respectively. We thank Sonia Ruiz-Piquer for her technical assistance in experiments with rats.

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Abbreviations

ACE, angiotensin I-converting enzyme; CSH, casein hydrolysate; GRAS, generally recognized as safe; LAB, lactic acid bacteria; LFH, lactoferrin hydrolysate; pCSH, casein hydrolysate permeate with molecular mass lower than 3 kDa; pLFH, lactoferrin hydrolysate permeate with molecular mass lower than 3kDa; RAS, renin angiotensin system; RP-HPLC, reversed phase-high performance liquid chromatography; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat; TFA, trifluoroacetic acid.

Dairy *Debaryomyces hansenii* strains produce the antihypertensive casein-derived peptides LHLPLP and HLPLP

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LWT - Food Science and Technology 61:

550-556 (2015)

ABSTRACT

The ability of dairy *Debaryomyces hansenii, Kluyveromyces lactis* and *Kluyveromyces marxianus* strains to release the casein-derived antihypertensive sequences RYLGY, AYFYPEL, LHLPLP, HLPLP, VPP and/or IPP was examined. Yeast strains were grown in medium with casein as sole nitrogen source and the yeast casein hydrolysates (CSHs) were analysed by HPLC-MS/MS to search for the six antihypertensive sequences. Only LHLPLP and HLPLP were identified in CSHs and exclusively in *D. hansenii* Dh1 and Dh14 hydrolysates in which both antihypertensive sequences represented approximately 6 (CSH Dh1) and 10% (CSH Dh14) of total peptide content. In addition, a complete analysis of selected CSHs by HPLC-MS/MS allowed the identification of 35 (Dh1) and 46 (Dh14) additional peptides, which could also contribute to the observed *in vitro* ACE inhibitory potency of both hydrolysates (Dh1, IC_{50} = 13.6 ± 1.8 μg/mL; Dh14, IC_{50} = 17.5 ± 2.1 μg/mL) and might confer them multifunctional properties. Finally casein zymography revealed the presence of at least one extracellular protease with a molecular mass of about 50 kDa. In conclusion, the present study contributes to a better insight into bioactive compounds produced by dairy yeasts and shows the feasibility of *D. hansenii* strains to produce antihypertensive casein-derived peptides.

Keywords: Dairy yeasts, *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, casein-derived antihypertensive peptides.

1. Introduction

Casein is an excellent substrate to produce peptides with angiotensin I converting enzyme (ACE)-inhibitory and antihypertensive effects (Otte, Shalaby, Zakora, Pripp, & El-Shabrawy 2007; Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012). Basically, processing of milk proteins with food grade proteolytic preparations or fermentation of milk with lactic acid bacteria (LAB) proteolytic starters have been employed to release antihypertensive peptides. Both approaches have conducted to the development of commercial products based on milk proteins with antihypertensive effects in humans. In fact, one of the most popular functional foods contain the casein-derived ACE-inhibitory tripeptides VPP and IPP, which can be obtained by means of either milk fermentation (Nakamura, Yamamoto, Sakai & Takano, 1995) or enzymatic hydrolysis using microbial proteases (Mizuno, Nishimura, Matsuura, Gotou & Yamamoto, 2004).

We have previously identified casein-derived sequences with antihypertensive activity using both methods. Two novel peptides of sequences RYLGY and AYFYPEL were identified from a peptic casein hydrolysate (Contreras, Carrón, Montero, Ramos, & Recio, 2009.). Both exerted *in vitro* inhibitory effects on ACE activity (IC_{50} values of 0.71 and 6.58 μM, respectively) and effectively decreased systolic blood pressure after oral administration to spontaneously hypertensive rats (SHRs) at 5 mg/kg of body weight. With respect to the fermentation strategy, the antihypertensive sequence LHLPLP was identified as one of the major peptides responsible for the ACE-inhibitory and antihypertensive effects of fermented milk produced by *Enterococcus faecalis* (Muguerza et al., 2006; Quirós et al., 2007). This sequence showed an IC_{50} value of 5.5 ± 0.4 μM against ACE activity and exhibited potent antihypertensive effect at a dose of 2 mg/kg (Quirós et al., 2007). Further characterization suggested the

pentapeptide HLPLP as the minimum active form responsible for the antihypertensive effect of LHLPLP (Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008.). Since the pathogenic potential of some *Enterococcus* strains (Franz, Holzapfel, & Stiles, 1999) may hamper their use in the food industry, production of HLPLP by enzymatic hydrolysis with food-grade enzymes was also optimized (Quirós, Hernández-Ledesma, Ramos, Martín-Álvarez, & Recio, 2012).

The impact of proteolytic yeast strains on the release of ACE-inhibitory peptides in fermented milk has been recently described (Chaves-López, Tofalo, Serio, Paparella, Sacchetti, & Suzzi, G, 2012; Chaves-López et al., 2014). We have shown the ability of some proteolytic dairy yeast strains belonging to *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Kluyveromyces marxianus* species to generate casein-derived antihypertensive hydrolysates (García-Tejedor, Padilla, Salom, Belloch, & Manzanares, 2013). With the aim of further characterize these non-conventional dairy yeasts as feasible GRAS (Generally Recognized As Safe) microorganisms for the production of antihypertensive peptides, here we screen up to thirty-three of these strains on the basis of their ability to release previously identified casein-derived antihypertensive sequences (RYLGY, AYFYPEL, LHLPLP, HLPLP, VPP and IPP) obtained either by enzymatic hydrolysis or bacterial fermentation. In addition, the presence of extracellular caseinolytic activity in selected strains is evaluated. Finally, quantification of the aforementioned sequences and identification of new main casein-derived peptides in the selected yeast casein hydrolysates are carried out, and their potential bioactivities in terms of ACE inhibition and other functional effects are discussed.

2. Materials and Methods

2.1 Materials

Casein (Promilk 85) was obtained from Ingredia (Arrax Cedex, France). ACE from porcine kidney, bicinchoninic acid protein assay kit, Hammarsten casein and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). Glucose was obtained from Panreac (Barcelona, Spain), bacteriological peptone was purchased from Cultimed (Barcelona, Spain) and yeast extract and agar were acquired from Pronadisa (Madrid, Spain). The fluorogenic substrate o-aminobenzoyl-Gly-p-nitro-Phe-Pro was provided by Bachem Feinchemikalien (Bubendorf, Switzerland). Coomassie Brilliant Blue G-250, 30% acrylamide/bis solution 29:1 and Precision Plus Protein Dual Color standards were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

2.2 Yeast strains and growth conditions

Thirty-three yeast strains belonging to *D. hansenii* (Dh1-Dh23), *K. lactis* (Kl1-Kl8) and *K. marxianus* (Km1-Km2) species isolated from artisanal ewes' and goats' milk cheeses (Padilla, Manzanares & Belloch, 2014) were used in this study. Yeast strains were maintained on GPYA medium (2% glucose, 0.5% peptone, 0.5% yeast extract and 2% agar, pH 5.5).

2.3 Preparation of casein hydrolysates

Stock solution of casein was sterilized by autoclaving at 121°C, 15 min. Casein medium (2% casein, 2% glucose) was inoculated with 10^6 cells/ml from pre-cultured strains on GPY (GPYA without agar) and incubated at 28°C and 100 rpm in an orbital shaker for 6 days. At the end of

the incubation period yeast cells were sedimented by centrifugation (3220 x g, 10 min), and the supernatants ultrafiltered using Amicon Ultra 10K centrifugal filter devices (Millipore Corporation, Billerica, MA, USA). Supernatant ultrafiltrates enriched in peptides of molecular weight lower than 10 kDa were considered as casein hydrolysates (CSHs). Supernatant concentrates (> 10 kDa) were used for zymography experiments as specified further.

Protein concentration was estimated by the bicinchoninic acid method using bovine serum albumin as standard (Ruiz-Giménez et al., 2012).

2.4 *In vitro* assay of ACE-inhibitory activity

In vitro ACE-inhibitory activity of CSHs was measured using the fluorescent method described by Sentandreu & Toldrá (2006) based on the hydrolysis of the internally quenched fluorescent substrate o-aminobenzoyl-Gly-p-nitro-Phe-Pro by the action of ACE.

The IC_{50} value of CSHs was defined as the protein 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 10.0 (SPSS Inc., Chicago, IL, USA).

2.5 Casein zymography

Zymography was performed according to Folio, Ritt, Alexandre, & Remize (2008) with minor modifications. Briefly, samples (casein supernatant concentrates) in loading buffer without β-mercaptoethanol were loaded onto a 10% (w/v) SDS/PAGE gel (Laemmli, 1970) co-

polymerized with 0.12% (w/v) Hammarsten casein. After electrophoresis and brief washing in ultrapure water, the gel was washed for 2 h on a rotary shaker with 2.5% (v/v) Triton X-100. Afterwards the gel was washed twice with reaction buffer (50 mM citrate phosphate buffer, pH 6) and then incubated overnight in reaction buffer at room temperature. The gel was stained with 0.1% (w/v) Coomassie Brilliant Blue G-250 for 1 h and then de-stained. Protease activity bands were visualized as white bands on a dark blue background.

2.6 Synthesis and quantification of peptides HLPLP and LHLPLP

The peptides HLPLP f(134-138) and LHLPLP f(133-138) from β-casein, were synthesized in-house using the fluorenyl-methoxy-carbonyl chloride (Fmoc) solid-phase method with a 431A peptide synthesizer (Applied Biosystems Inc. Überlingen, Germany). Peptide purity was calculated by reversed phase high performance liquid chromatography-UV and mass spectrometry (RP-HPLC-UV-MS).

Six-point calibration curves of the pure peptides HLPLP and LHLPLP (from 1.95 to 62.5 µg/mL) were prepared in Milli-Q deionized water. Duplicate injections were done for each point on the calibration curve. The quantification of the peptides in each CSH was performed by the extraction of their characteristic ions.

2.7 Peptide sequencing by reversed phase-high-performance liquid chromatography tandem mass spectrometry (RP-HPLC-MS/MS).

The analyses of CSHs by RP-HPLC-MS/MS were performed on an Agilent HPLC (Agilent Technologies, Waldbronn, Germany) system followed by on-line MS/MS analysis on a quadrupole ion trap instrument (Esquire 3000, Bruker Daltonik GmbH, Bremen, Germany) as previously

described (Sánchez-Rivera, Diezhandino, Gómez-Ruiz, Fresno, Miralles, & Recio, 2014). Chromatographic separations were performed with a Mediterranea Sea_{18} 150 mm × 2.1 mm column (Teknokroma, Barcelona, Spain). The flow rate was 0.2 mL/min and the injection volume 50 μL. Peptides were eluted with a linear gradient from 0% to 45% of solvent B (acetonitrile:formic acid 0.1%) and 55% solvent A (water:formic acid 0.1%) in 120 min. The target mass was set at *mass-to-charge* ratio (*m/z*) 750, and the mass acquisition ranged from 200 to 2000 *m/z*.

2.8 Statistical data analysis

IC_{50} values of CSHs were compared using Student's t-test. Differences with *P*-values < 0.05 were considered significant. Data statistical analysis was performed using the GraphPad Prism 4 software (GraphPad Software Inc, La Jolla, CA, USA).

3. Results and discussion

Non-Saccharomyces yeasts have been utilized as industrial organisms for a variety of biotechnological roles since numerous and diverse biological activities make them promising candidates for a wide range of applications not limited to the food sector. They are increasingly being used as hosts for expression of proteins, biocatalysts and multienzyme pathways for the synthesis of fine chemicals and small molecular weight compounds of medicinal and nutritional importance (Johnson, 2013).

In the present study, 33 CSHs generated by *D. hansenii* (CSH Dh1-Dh23) *K. lactis* (CSH Kl1-Kl8) and *K. marxianus* (CSH Km1-Km2) strains isolated from artisanal cheeses (Padilla et al., 2014) were analysed by HPLC-MS/MS to search for the previously identified antihypertensive sequences RYLGY, AYFYPEL, LHLPLP, HLPLP, VPP and/or IPP. Peptides RYLGY, AYFYPEL, VPP and IPP were not identified in any of the CSHs. However, peptide HLPLP and its precursor sequence LHLPLP were released from casein after growth of *D. hansenii* strains Dh1 and Dh14. Figure 1 shows the UV chromatogram and the extracted ions corresponding to both peptides.

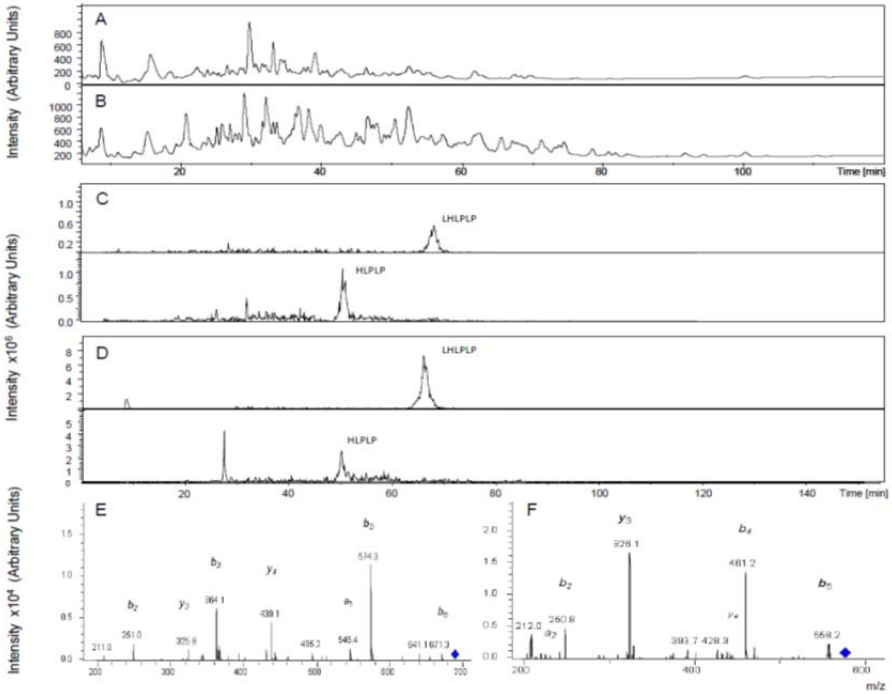

Figure 1. UV chromatogram of the casein hydrolysate Dh1 (A) and Dh14 (B). Extracted Ion chromatogram (EIC) of the peptides LHLPLP and HLPLP in Dh1 (C) and Dh14 (D). Fragmentation pattern of LHLPLP (E) and of HLPLP (F), with mass-to-charge ratios (m/z) of 689.5 and 576.3, respectively.

Quantification of HLPLP and LHLPLP in CSH Dh1 and Dh14 was carried out using linear regressions obtained by HPLC-MS/MS. As shown in Table 1, highest total content was obtained in CSH Dh14. However both hydrolysates contained similar quantities of the pentapeptide. These antihypertensive peptides represented approximately 6 and 10% of total peptide content in Dh1 and Dh14 hydrolysates, respectively. No significant differences were found between the ACE inhibitory potencies of both *D. hansenii* CSHs, which showed IC_{50} values of 13.6 ± 1.8 μg/mL (Dh1) and 17.5 ± 2.1 μg/mL (Dh14). ACE inhibition was slightly higher than the previously reported values of milk fermented by *E. faecalis* (34-59 μg/mL; Muguerza et al., 2006) but lower than that showed by a pepsin casein hydrolysate (5.68 μg/mL; Contreras et al., 2009).

Table 1. Content of peptides LHLPLP and HLPLP in casein hydrolysates (CSH) Dh1 and Dh14.

CSH	LHLPLP (μg/mg)	HLPLP (μg/mg)	Total (μg/mg)
Dh1	15.6	43.5	59.1
Dh14	52.7	46.4	99.1

During milk fermentation, proteolysis by LAB has been studied in detail. The key enzyme in this process is a cell-envelope proteinase involved in the first step of casein degradation (Savijoki, Ingmer, & Varmanen, 2006). By contrast, although yeast caseinolytic activity is well documented few reports deal with the characterization of the extracellular proteolytic system. With regards to *D. hansenii*, Kumura, Takagaki, Sone, Tsukahara, Tanaka, & Shimazaki (2002) reported that a cell-wall associated protease acted on β-casein whereas α_S-casein degradation was

attributed to an intracellular protease released by cell lysis. However, extracellular proteolytic activity on both α_S- and β-casein for *D. hansenii* strains isolated from cheese has been described (Gardini et al., 2006). Here we conducted a casein zymography to detect extracellular proteases in the supernatants of the two selected *D. hansenii* strains. On casein substrate, extracellular proteolytic activities were detected (Figure 2). Analysis of Dh14 supernatant revealed the presence of one hydrolysis band which corresponded to a molecular mass of about 50 kDa. The same band was observed in Dh1 supernatant, in which a second band of slightly higher molecular mass was also detected. Band intensities for both supernatants were not related to the amount of total protein loaded (Dh1, 4.8 µg; Dh14, 4.4 µg). Although increasing amounts of Dh14 total protein were loaded onto SDS/PAGE gels for protease detection no other clear activity bands were observed (data not shown). These results might suggest a different proteolytic profile for both strains in the conditions tested. Future work should include purification of the extracellular *D. hansenii* enzymes and determination of its biochemical properties.

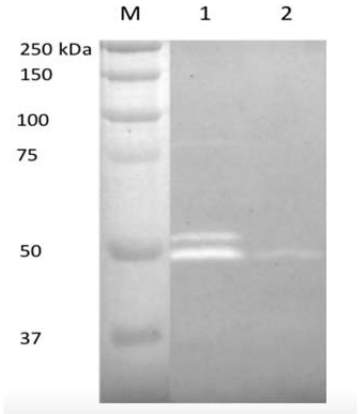

Figure 2. Casein zymography of *D. hansenii* Dh1 and Dh14 extracellular proteolytic activities. Lane M, molecular weight markers (Precision Plus Protein Dual Color standards, Bio-Rad); lane 1, Dh1 culture supernatant concentrate (4.8 µg); lane 2, Dh14 culture supernatant concentrate (4.4 µg).

Over the last two decades, a large number of casein-derived peptides obtained by fermentation and enzymatic hydrolysis with ACE-inhibitory potency and proven antihypertensive effects in SHRs have been identified (Martínez-Maqueda et al., 2012). Although LHLPLP and HLPLP might contribute to the inhibitory potency of *D. hansenii* CSHs, other sequences generated from casein hydrolysis might also be responsible for the observed inhibitory effects. This prompted us to carry out a complete analysis of CSH Dh1 and Dh14 by HPLC-MS/MS, which allowed the identification of 35 and 46 additional peptides (Table 2 and 3), respectively. The most abundant peptides in each hydrolysate are given in bold. As can be seen in Table 2, most of the peptides present in CSH Dh1, derived from hydrolysis of α_{S1}-CN (15 fragments) and β-CN (14 fragments) whereas only 6 and 2 peptides corresponded to α_{S2}-CN and κ-CN fragments, respectively. By contrast, in CSH Dh14 most of the peptides (37 fragments) derived from β-CN. In addition 9 fragments from α_{S1}-CN and 2 from κ-CN were also identified, while none of the fragments derived from α_{S2}-CN. Finally, only 11 of the peptides identified, including the two antihypertensive sequences LHLPLP and HLPLP, were common for both *D. hansenii* strains, which indicates strain-specific proteolysis in accordance with the results obtained in the zymogram and also as described for the *Streptococcus thermophilus* cell envelope proteinase (Miclo et al., 2012).

Table 2. Identification of peptides contained in *D. hansenii* casein hydrolysate (CSH) Dh1.

Ion for MS/MS (m/z)[a]	Observed mass[b]	Theoretical mass	Protein fragment	Identified sequence[c]
806.388	805.372	805.401	α_{S1}-CN f(24-30)	**FVAPFPE**
530.179	529.172	529.29	α_{S1}-CN f(25-29)	**VAPFP**
659.312	658.304	658.333	α_{S1}-CN f(25-30)	**VAPFPE**
608.373	607.366	607.333	α_{S1}-CN f(32-36)	FGKEK
618.394	617.387	617.302	α_{S1}-CN f(35-39)	EKVNE
632.362	631.354	631.318	α_{S1}-CN f(81-85)	IQKED
502.15	501.143	501.28	α_{S1}-CN f(95-98)	LEQL
468.196	467.189	467.249	α_{S1}-CN f(127-130)	IHAQ
602.321	601.313	601.307	α_{S1}-CN f(138-142)	VNQEL
680.309	679.302	679.297	α_{S1}-CN f(159-164)	YPSGAW
517.184	516.177	516.233	α_{S1}-CN f(160-164)	PSGAW
777.435	776.428	776.407	α_{S1}-CN f(167-173)	VPLGTQY
581.257	580.25	580.286	α_{S1}-CN f(169-173)	**LGTQY**
555.344	554.337	554.27	α_{S1}-CN f(181-185)	**DIPNP**
519.164	518.156	518.234	α_{S1}-CN f(186-190)	IGSEN
602.321	601.313	601.253	α_{S2}-CN f(4-8)	MEHVS
1000.532	999.525	999.539	α_{S2}-CN f(100-108)	YQGPIVLNP
837.459	836.452	836.476	α_{S2}-CN f(101-108)	QGPIVLNP
698.394	697.386	697.401	α_{S2}-CN f(116-122)	**AVPITPT**
528.248	527.24	527.296	α_{S2}-CN f(118-122)	**PITPT**
720.417	719.409	719.422	α_{S2}-CN f(198-203)	**TKVIPY**
628.27	627.263	627.323	β-CN f(6-11)	LNVPGE
748.265	747.258	747.304	β-CN f(38-43)	QQQTED
622.309	621.302	621.316	β-CN f(59-63)	**VYPFP**
439.026	438.018	438.284	β-CN f(74-77)	**IPPL**
668.38	667.373	667.39	β-CN f(74-79)	IPPLTQ
732.347	731.34	731.385	β-CN f(113-118)	KYPVEP
690.282	689.275	689.323	β-CN f(127-132)	LTDVEN
577.164	576.157	576.239	β-CN f(128-132)	TDVEN
689.449	688.442	688.427	β-CN f(133-138)	**LHLPLP**
576.304	575.297	575.343	β-CN f(134-138)	**HLPLP**
649.265	648.258	648.28	β-CN f(144-148)	MHQPH
805.351	804.343	804.384	β-CN f(155-161)	**VMFPPQS**
780.457	779.45	779.491	β-CN f(170-176)	VLPVPQK
536.148	535.14	535.228	β-CN f(193-196)	YQEP
633.35	632.343	632.353	k-CN f(26-30)	IPIQY
663.318	662.311	662.339	k-CN f(43-47)	YQQKP

[a]Charge of precursor ion: 1
[b]Calculated monoisotopic mass.
[c]Main peptides are labelled in bold.
Casein: CN

Table 3. Identification of peptides contained in *D. hansenii* casein hydrolysate (CSH) Dh14.

Ion for MS/MS (m/z)[a]	Observed mass[b]	Theoretical mass	Protein fragment	Identified sequence[c]
805.399	805.378	805.401	α_{S1}-CN f(24-30)	**FVAPFPE**
676.407	675.400	675.359	α_{S1}-CN f(29-34)	PEVFGK
707.446	706.439	706.401	α_{S1}-CN f(31-36)	VFGKEK
763.321	762.322	762.358	α_{S1}-CN f(54-59)	MEDIKQ
732.394	631.328	631.318	α_{S1}-CN f(55-59)	EDIKQ
903.472	902.465	902.453	α_{S1}-CN f(135-142)	MIGVNQEL
517.171	516.164	516.233	α_{S1}-CN f(160-164)	PSGAW
789.371	788.634	788.370	α_{S1}-CN f(179-185)	FSDIPNP
730.385	729.314	729.329	α_{S1}-CN f(184-190)	NPIGSEN
675.257	674.322	674.324	β-CN f(1-5)	RELEE
649.284	627.306	627.323	β-CN f(6-11)	LNVPGE
633.295	632.288	632.277	β-CN f(38-42)	QQQTE
748.284	747.277	747.304	β-CN f(38-43)	QQQTED
875.371	876.305	876.346	β-CN f(38-44)	QQQTEDE
632.355	631.335	631.318	β-CN f(44-48)	ELQDK
681.303	680.296	680.313	β-CN f(52-57)	FAQTQS
622.309	621.302	621.316	β-CN f(59-63)	VYPFP
668.416	667.409	667.390	β-CN f(74-79)	IPPLTQ
769.342	768.386	768.438	β-CN f(74-80)	IPPLTQT
866.472	865.465	865.491	β-CN f(74-81)	**IPPLTQTP**
753.385	752.378	752.407	β-CN f(75-81)	PPLTQTP
689.313	688.395	688.412	β-CN f(95-100)	**VSKVKE**
760.456	759.449	759.449	β-CN f(95-101)	VSKVKEA
577.327	576.267	576.294	β-CN f(98-102)	VKEAM
651.384	650.376	650.343	β-CN f(111-115)	FPKYP
732.274	731.364	731.385	β-CN f(113-118)	KYPVEP
611.329	610.267	610.260	β-CN f(119-123)	FTESQ
698.269	697.262	697.292	β-CN f(119-124)	**FTESQS**
904.446	903.439	903.455	β-CN f(125-132)	**LTLTDVEN**
689.313	688.392	688.427	β-CN f(133-138)	**LHLPLP**
802.505	801.498	801.511	β-CN f(133-139)	LHLPLPL
1130.667	1129.660	1129.686	β-CN f(133-142)	LHLPLPLLQS
576.334	575.327	575.343	β-CN f(134-138)	**HLPLP**
793.521	792.514	792.511	β-CN f(135-141)	LPLPLLQ
880.515	879.508	879.543	β-CN f(135-142)	**LPLPLLQS**
670.420	669.413	669.406	β-CN f(137-142)	LPLLQS
533.184	532.177	532.265	β-CN f(140-143)	LQSW
649.329	648.277	648.280	β-CN f(144-148)	MHQPH
805.402	804.348	804.384	β-CN f(155-161)	**VMFPPQS**
649.327	648.320	648.344	β-CN f(164-169)	SLSQSK
543.270	542.262	542.343	β-CN f(168-172)	SKVLP
559.253	558.246	558.284	β-CN f(185-189)	**MPIQA**
762.385	761.378	761.396	β-CN f(191-196)	LLYQEP
649.318	648.310	648.312	β-CN f(192-196)	LYQEP
826.445	825.438	825.450	β-CN f(199-206)	**GPVRGPFP**
939.500	938.493	938.534	β-CN f(199-207)	**GPVRGPFPI**
658.370	657.363	657.370	k-CN f(119-124)	IPTINT
757.362	756.355	756.365	k-CN f(155-161)	**SPPEINT**

[a]Charge of precursor ion: 1
[b]Calculated monoisotopic mass.
[c]Main peptides are labeled in bold.
CN: casein

Sequences LHLPLP and HLPLP were also included in several longer fragments identified in CSH Dh14, such as LHLPLPL, HLPLPL and LHLPLPLLQS. The heptapeptide LHLPLPL, with an IC_{50} value of 425 ± 44 μM against ACE, was also identified in *E. faecalis* fermented milk (Quirós et al., 2007). Another peptide (β-CN f(58-76), LVYPFPGPIPNSLPQNIPP; IC_{50} = 5.2 ± 0.3 μM) identified in *E. faecalis* fermented milk caused also significant decrease of the systolic blood pressure in SHRs (Quirós et al., 2007). Part of this sequence (VYPFP) was also identified in both *D. hansenii* CSHs. On the other hand, although neither VPP nor IPP were found in *D. hansenii* CSHs, sequences containing the latter at the N-terminal end were identified (IPPL, IPPLTQ, IPPLTQT and IPPLTQTP). Whether these newly identified sequences could also contribute to the ACE-inhibitory activity of CSH Dh1 and Dh14 deserves further characterization studies. Moreover, the role of gastrointestinal digestion to generate active fragments from longer sequences should also be taken into account, although degradation of active fragments can also occur.

More than 60% of the identified sequences have at least one Pro residue. Particularly, Pro in the C-terminal and antepenultimate position has been recognised as one of the most favourable residues for peptide binding to the active site of ACE (Rohrbach, Willians, & Rolstad, 1981). Interestingly, in addition to the antihypertensive sequences LHLPLP and HLPLP, peptides VAPFP, DIPNP, VYPFP, FSDIPNP and GPVRPFP with a Pro residue in both positions might be promising candidates to inhibit ACE activity. Finally, some other sequences identified in CSH Dh1 and Dh14 were previously characterized as ACE-inhibitory and antioxidant peptides (sequence YQEP in Dh1; Silva, Pihlanto, & Malcata, 2006) or having lipoxygenase inhibitory properties (VLPVPQK in Dh1; Rival, Fornaroli, Boeriu, & Wichers, 2001). Also some sequences identified here share structure homology with fragments able to inhibit prolyl-peptidases of

human colon cells (FVAPFPE, VAPPFP and VAPFPE in Dh1; Juillerat-Jeanneret, Robert, & Juillerat, 2011) or having immune-modulating and ACE-inhibitory properties (GPVRGPFPI and GPVRGPFP in Dh14; Hayes, Stanton, FitzGerald, & Ross, 2007; Hernández-Ledesma, Amigo, Ramos, & Recio, 2004). The multifunctional properties and the resulting health benefits of dairy protein hydrolysates have been pointed out by several studies (reviewed in Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014). To what extent *D. hansenii* CSHs might exert different functional effects requires further research.

D. hansenii is a highly heterogeneous, and thus versatile, species which represents an attractive target for fundamental and applied biotechnological research (Johnson, 2013; Hatoum et al., 2013). Our results point to dairy *D. hansenii* strains Dh1 and Dh14 as feasible GRAS microorganisms with strain-specific caseinolytic systems for the production of the antihypertensive sequences HLPLPL and HLPLP. These two peptides could at least in part be responsible for the ACE-inhibitory properties of both *D. hansenii* CSHs, although data reported here suggest that other potential bioactive sequences are also produced. *D. hansenii,* one of the predominant yeast species in all type of cheeses, plays important roles in cheese making and further investigation is needed to exploit its biotechnological potential.

4. Acknowledgements

This work was supported by grants AGL2010-21009 and AGL2011-24643 from 'Ministerio de Educación y Ciencia - FEDER', Consolider Ingenio 2010, Fun-C-Food, CSD2007-00063 and RETICS INVICTUS RD12/0014/0004 from 'Instituto de Salud Carlos III'. A. García-Tejedor and L. Sánchez-Rivera are recipients of predoctoral fellowships from 'Ministerio de Educación y Ciencia' (BES-2011-044424) and CSIC (JAE-PreDoc), respectively. Dairy yeast isolates were kindly provided by Carmela Belloch (IATA-CSIC).

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Abbreviations

ACE, angiotensin I-converting enzyme; CSH, casein hydrolysate; GRAS, generally recognized as safe; LAB, lactic acid bacteria; RP-HPLC-MS/MS, reversed phase-high performance liquid chromatography tandem mass spectrometry; RP-HPLC-UV-MS, reversed-phase high performance liquid chromatography-UV and mass spectrometry; SHR, spontaneously hypertensive rat.

Objetivo II

Capítulo III. Novel antihypertensive lactoferrin-derived peptides produced by *Kluyveromyces marxianus*: Gastrointestinal stability profile and *in vivo* angiotensin I-converting enzyme (ACE) Inhibition. *Journal of Agricultural and Food Chemistry* 62: 1609−1616 (2014).

Capítulo IV. *In vivo* antihypertensive mechanism of lactoferrin-derived peptides: reversion of angiotensin I- and angiotensin II-induced hypertension in Wistar rats. *Journal of Functional Foods*. 15: 294-300 (2015).

Capítulo V. An antihypertensive lactoferrin hydrolysate inhibits angiotensin I-converting enzyme, modifies expression of hypertension-related genes and enhances nitric oxide production in cultured human endothelial cells. *Journal of Functional Foods.* 12: 45-54 (2015).

Novel Antihypertensive Lactoferrin-Derived Peptides Produced by *Kluyveromyces marxianus*: Gastrointestinal Stability Profile and *in vivo* Angiotensin I-Converting Enzyme (ACE) Inhibition

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Journal of Agricultural and Food Chemistry 62:

1609−1616 (2014)

ABSTRACT

Novel antihypertensive peptides released by *Kluyveromyces marxianus* from bovine lactoferrin (LF) have been identified. *K. marxianus* LF permeate was fractionated by semi-preparative high performance liquid chromatography and 35 peptides contained in the angiotensin I converting enzyme (ACE)-inhibitory fractions were identified by using an ion trap mass spectrometer. Based on peptide abundance and common structural features, six peptides were chemically synthesized. Four of them (DPYKLRP, PYKLRP, YKLRP and GILRP) exerted *in vitro* inhibitory effects on ACE activity and effectively decreased systolic blood pressure after oral administration to spontaneously hypertensive rats (SHRs). Stability against gastrointestinal enzymes suggested that the sequence LRP could contribute to the *in vivo* effects of parental peptides. Finally, there were reductions in circulating ACE activity and angiotensin II level in SHRs after either DPYKLRP or LRP intake, thus confirming ACE inhibition as *in vivo* mechanism for their antihypertensive effect.

Keywords: *Kluyveromyces marxianus*, lactoferrin-derived peptides, gastrointestinal digestion, antihypertensive effect, *in vivo* ACE inhibition.

1. Introduction

In the last decade much work has been done to characterize the antihypertensive effects of peptides derived from food proteins.[1] Angiotensin I-converting enzyme (ACE) inhibition is the main target for those peptides. ACE, as part of the renin-angiotensin system (RAS), hydrolyzes both the inactive angiotensin I into vasoconstrictor angiotensin II and the vasodilator bradykinin into an inactive peptide leading to blood pressure upregulation.[2] *In vitro* inhibitory effect of food protein derived peptides on ACE activity is well established in contrast with the limited *in vivo* evidence available for the mechanism of action underlying their blood pressure lowering effect. Also bioavailability of ACE-inhibitory peptides has been intensively studied since it is known that bioactive peptides may undergo physiological transformations that determine their activity in the organism.[3] Most research has been focused on milk derived antihypertensive peptides, some of which have shown beneficial effects in clinical assays, as reported in different meta-analyses.[4]

The use of the proteolytic system of lactic acid bacteria (LAB) to hydrolyze milk proteins is a successful strategy to release antihypertensive peptides.[5] By contrast few studies exploit the proteolytic potential of yeasts despite their contribution to proteolysis in dairy products is well established. In this context, the lactose-fermenting yeast *Kluyveromyces marxianus* regularly found in milk and dairy products has been pointed out as a promise candidate to generate antihypertensive peptides from the whey proteins α-lactalbumin and β-lactoglobulin.[6] Its potential to produce fermented milk with casein-derived ACE-inhibitory peptides has been also described[7] although *in vivo* antihypertensive effects were not evaluated in any of these reports.

Bovine lactoferrin (LF), a well-characterized component of milk whey, is also a good source of antihypertensive peptides. We have shown that enzymatic LF hydrolyzates lower blood pressure and thus exhibit potential as orally effective antihypertensive compounds.[8,9] Moreover, after long-term intake of a pepsin LF hydrolyzate, there were reductions of circulating ACE activity, angiotensin II and aldosterone levels, as well as a compensatory increase of renin activity.[10] So far, only five LF-derived peptides with sequences RRWQWR, WQ[11], RPYL, LIWKL and LNNSRAP[8] have shown antihypertensive effects after oral administration to spontaneously hypertensive rats (SHRs), although based on *in silico* studies some other antihypertensive peptides are expected to be still identified and isolated from LF hydrolyzates.[12]

In a previous work, proteolytic yeast strains of *Debaryomyces hansenii*, *Kluyveromyces lactis* and *K. marxianus* isolated from cheeses[13] were screened for their ability to grow in media with LF as sole nitrogen source and to produce LF hydrolyzates containing ACE-inhibitory peptides. *K. marxianus* Km2 strain grown on LF produced the most potent hydrolyzate which, when orally administered to SHRs, exerted antihypertensive effect.[14]

The objective of the present study was to identify the LF-derived peptides produced by *K. marxianus* Km2 and characterize their antihypertensive effects. For this purpose a *K. marxianus* LF permeate enriched in peptides of molecular weight lower than 3 kDa (pLFH) was fractionated and the main peptides present in the ACE-inhibitory fractions identified by using an ion trap mass spectrometer. Selected peptides were evaluated for their inhibitory effects on ACE activity, their antihypertensive effects in SHRs and their stability against simulated gastrointestinal digestion. Finally the *in vivo* effect of peptides on SHRs blood ACE activity as well as angiotensin II and aldosterone levels are discussed.

2. Materials and Methods

2.1 Materials

Bovine LF was provided by FrieslandCampina Domo (Zwolle, The Netherlands). ACE from porcine kidney, captopril, and bicinchoninic acid protein assay kit were purchased from Sigma (St. Louis, MO). Glucose was obtained from Panreac (Barcelona, Spain), bacteriological peptone was purchased from Cultimed (Barcelona, Spain) and yeast extract and agar were acquired from Pronadisa (Madrid, Spain). ACE substrate o-aminobenzoylglycyl-p-nitrophenylalanylproline was provided by Bachem Feinchemikalien (Bubendorf, Switzerland). Corolase PP (porcine pancreatic extract) was from AB enzymes (Darmstadt, Germany). Diazepam and ketamine were purchased from Roche Farma (Madrid, Spain) and Parke-Davis (Alcobendas, Madrid, Spain), respectively. ACE colorimetric kit was acquired from Bühlmann Laboratories (Schönenbuch, Switzerland). AssayMax Angiotensin II ELISA kit was from AssayPro (Saint Charles, MI) and Coat-A-Count Aldosterone ^{125}I RIA kit was provided by Siemens Medical Solutions Diagnostics (Los Angeles, CA).

2.2 Preparation of *K. marxianus* lactoferrin permeate (pLFH) and fractionation by reversed-phase high-performance liquid chromatography (RP-HPLC)

K. marxianus LF hydrolyzate was prepared as previously described and it was subjected to ultrafiltration through a VivaFlow 50 3kDa cut-off polyethersulfone membrane (Vivascience, Sartorius Stedim Biotech, Aubagne, France). Resulting permeate (pLFH), enriched in peptides of molecular weight lower than 3 kDa showed an IC_{50} value of 50.2 ± 2.7 µg/mL. [14]

Fractionation of pLFH was carried out by RP-HPLC using a Waters system (Waters Corporation, Milford, MA) equipped with a 1525 Binary HPLC pump, a 2996 Photodiode Array Detector and a 717 plus Autosampler in combination with a Fraction Collector III. For this purpose, pLFH was applied to a Prep Nova-Pak® HR C18, 60 Å, 6 µm, 7.8 x 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 20% B in 70 min. Samples of the whole permeate and the fractions (20 mL) were freeze-dried and kept at -20ºC until reconstitution with distilled water for determination of the protein content and *in vitro* ACE-inhibitory effect, as explained below.

2.3 Peptide sequencing by reversed-phase high-performance liquid chromatography tandem mass spectrometry (RP-HPLC-MS/MS)

RP-HPLC-MS/MS analysis of pLFH fractions was performed as described by Sánchez-Rivera et al.[15] with minor changes. The flow rate was 0.2 mL/min and the injection volume 50 µL. Peptides were eluted using a linear gradient from 0 to 45% of solvent B (acetonitrile:formic acid; 1,000:0.1, v/v) and 55% of solvent A (water:formic acid; 1,000:0.1% v/v) in 120 min. Data Analysis (version 4.0; Bruker Daltoniks) was used to process and transform spectra to representing mass values. BioTools (version 3.2; Bruker Daltoniks) was used to process the MSn spectra, to perform peptide sequencing and to calculate theoretical masses.

Main peptides identified in the pLFH 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.

2.4 *In vitro* assay of ACE-inhibitory activity

In vitro ACE-inhibitory activity of pLFH fractions and synthetic peptides was measured using the fluorescent method described by Sentandreu and Toldrá[16] based on the hydrolysis of the internally quenched fluorescent substrate o-aminobenzoylglycyl-p-nitrophenylalanylproline by the action of ACE. Protein content of peptide fractions was estimated by the bicinchoninic acid method (BCA) using bovine serum albumin as standard.[7] Synthetic peptide concentration was based on the dry weight of the peptides.

The IC_{50} value was defined as the protein/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 10.0 (SPSS Inc., Chicago, IL).

2.5 *In vivo* assay of antihypertensive effect in SHRs

Experimental procedures were conducted in accordance with 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. The study was approved by the 'Ethics Committee for Animal Welfare' of 'La Fe' Hospital to be carried out in its accredited animal research facility.

Male SHRs weighing 230–330 g (Charles River Laboratories, Barcelona, Spain) were housed in temperature-controlled rooms (23°C) with 12 h light/dark cycles and consumed tap water and standard diets *ad libitum*. To minimize the impact of light cycle and feeding on circadian rhythms of blood pressure,[17] the experiments started always at the same time in the morning (9:00 a.m.) in fasted rats. Indirect measurement of

systolic blood pressure (SBP) was carried out in eighteen awake restrained rats by the non-invasive tail-cuff method using computer-assisted Non-Invasive Blood Pressure equipment (LE5001 unit with LE5160R cuff & transducer, Panlab Harvard Apparatus, Cornellá, Barcelona, Spain). Peptides (up to 10 mg/kg) were orally administered by gastric intubation in 650 µL of physiological saline. Before the measurements, rats were kept at 37°C during 15 min to make the pulsations of the tail artery detectable. The SBP was measured before peptide intake (zero time) and 1, 2, 3, 4 and 24 h after intake. Physiological saline (650 µL) and captopril (50 mg/kg) served as negative and positive controls, respectively. Each value of SBP was obtained by averaging at least three consecutive and successful measurements without disturbance of the signal. Changes in SBP were calculated as the absolute difference (in mm Hg) with respect to the basal values of measurements obtained just before peptide administration.

2.6 *In vitro* simulated gastrointestinal digestion and analysis of digests by RP-HPLC

Peptides were subjected to a two-stage simulated gastrointestinal digestion process as previously described.[10] Briefly, pepsin (0.2 mg) was added to aqueous solutions of peptides (10 mL; 1 mM) adjusted at pH 2.0 using 1 N HCl and incubated at 37°C. After 90 min, the pH was adjusted to 7.5 adding 10 mL of 0.4 M sodium phosphate buffer at pH 7.5. Corolase PP, a proteolytic enzyme preparation that contains trypsin, chymotrypsin, and amino and carboxypeptidase activities, was added (0.2 mg), and the sample was further incubated at 37°C for 150 min. The reaction was stopped by heating at 80ºC for 10 min in a water bath, followed by cooling at room temperature. Each sample was stored at -20°C until further analysis by RP-HPLC.

Analysis of gastrointestinal digests was performed in the same RP-HPLC system specified above using a Symmetry C18 column (4.6 × 150 mm, 5 µm, Waters) kept at 40°C. The column was developed at a flow rate of 1 mL/min. Peptides were eluted with a linear gradient of solvent B (acetonitrile with 0.1% TFA) in solvent A (water with 0.1% TFA) from 0 to 40% in 20 min and detected at 214 nm. Peptides LRP and KLRP were quantified in gastrointestinal digests of DPYKLRP, PYKLRP and YKLRP in accordance to standard curves in water.

2.7 Determination of blood components of the renin-angiotensin system

Twenty-two rats were anaesthetized by intraperitoneal injection of 5 mg/kg diazepam and 100 mg/kg ketamine. Blood samples were collected from the abdominal aorta to obtain both serum and plasma which were kept frozen at -80ºC until the determination of ACE activity, angiotensin II and aldosterone levels.

Direct quantitative *in vitro* determination of ACE activity was carried out by using the Bühlmann ACE colorimetric kit according to the manufacturer's instructions. Briefly, it is a kinetic enzymatic assay in which ACE catalyses the cleavage of the synthetic substrate (FAPGG) into an amino acid derivative and a dipeptide. The kinetic of this cleavage reaction is measured by recording the decrease in absorbance at 340 nm.

Quantitative *in vitro* measurement of angiotensin II was carried out by using the AssayMax Angiotensin II ELISA kit according to the manufacturer's instructions. Briefly, this assay employs a quantitative sandwich enzyme immunoassay technique in which a polyclonal antibody specific for angiotensin II is pre-coated onto a microplate. The angiotensin II in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for angiotensin II, which is

recognized by a streptavidin-peroxidase conjugate. A peroxidase enzyme substrate is added and intensity of developed color is measured.

Quantitative *in vitro* measurement of aldosterone was carried out by using the Coat-A-Count Aldosterone ^{125}I RIA kit according to the manufacturer's instructions. Briefly, it is a solid-phase radioimmunoassay, based on aldosterone-specific antibody immobilized to the wall of the assay tube. ^{125}I-labelled aldosterone competes for a fixed time with aldosterone in the sample for antibody sites.

3. Results

3.1 Fractionation of *K. marxianus* pLFH: ACE-inhibitory activity of resulting fractions and identification of major peptides

K. marxianus pLFH was subjected to semi-preparative RP-HPLC and the total chromatogram was divided into 11 fractions which showed IC_{50} values ranging from 49 to 288 μg/mL. The three most active fractions (F6, F7 and F11) with IC_{50} values of 68, 74 and 49 μg/mL, respectively, were analyzed by HPLC-MS/MS and the major peptide components were sequenced (35 peptides on total, Table 1).

Table 1. Identification of Peptides Contained in the F6, F7 and F11 RP-HPLC Fractions of the *K. marxianus* Lactoferrin Permeate (pLFH)

Fraction	Ion for MS/MS (m/z)[a]	Observed mass[b]	Theoretical mass	Protein fragment	Identified sequence
F6	755.397	754.390	754.372	f(601 – 607)	SDRAAHV
	779.362	778.354	778.361	f(652 – 658)	GGRPTYE
	646.390	645.382	645.333	f(335 – 340)	AEEVKA
	676.329	675.322	675.308	f(261 – 266)	**DGKEDL**[c]
	624.367	623.360	623.303	f(283 – 287)	SRSFQ
	638.438	637.431	637.355	f(611 – 615)	LLHQQ
	668.412	667.405	667.340	f(602 – 607)	DRAAHV
	607.349	606.341	606.276	f(68 – 72)	GRDPY
	439.113	438.105	438.211	f(319 – 322)	YLGS
	722.407	721.400	721.376	f(276 – 281)	EKFGKN
F7	908.426	907.419	907.404	f(652 – 659)	GGRPTYEE
	575.214	574.206	574.260	f(536 – 541)	DVGDVA
	714.516	713.509	713.480	f(435 – 441)	AVAVVKK
	775.417	774.409	774.376	f(260 – 266)	VDGKEDL
	504.075	503.068	503.223	f(536 – 540)	DVGDV
	851.407	850.400	850.382	f(653 – 659)	GRPTYEE
	548.256	547.249	548.226	f(503 – 508)	ALCAGD
	636.454	635.447	635.375	f(338 – 342)	VKARY
	582.226	581.219	581.270	f(660 – 664)	YLGTE
	572.331	571.324	571.333	f(28 – 33)	KLGAPS
	496.155	495.148	495.244	f(283 – 286)	SRSF
	779.466	778.459	778.434	f(98 – 104)	VKKGSNF
	861.351	860.344	860.366	f(86 – 92)	**ESPQTHY**[c]
F11	848.427	847.420	847.455	f(68 – 74)	GRDPYKL
	823.472	822.465	822.387	f(224 – 229)	RDQYEL
	693.239	692.232	692.281	f(189 – 194)	YFGYSG
	743.382	742.375	742.386	f(141 – 147)	SLEPLQG
	773.499	772.492	772.460	f(71 – 76)	**PYKLRP**[c]
	907.415	906.408	906.420	f(563 – 569)	NLNREDF
	780.312	779.305	779.345	f(101 – 107)	GSNFQLD
	677.293	676.286	676.318	f(445 – 450)	GLTWNS
	676.510	675.503	675.407	f(72 – 76)	**YKLRP**[c]
	555.428	554.421	554.354	f(130 – 134)	**GILRP**[c]
	888.482	887.475	887.487	f(70 – 76)	**DPYKLRP**[c]
	414.156	413.149	413.227	f(144 – 147)	PLQG

[a]Charge of precursor ion: 1
[b]Calculated monoisotopic mass
[c]Chemically synthesized peptides are labelled in bold.

3.2 ACE-inhibitory activity of LF-derived peptides

A total of 6 peptides (labeled in Table 1) from those identified in fractions F6, F7 and F11 were chemically synthesized. These included four sequences (DGKEDL, ESPQTHY, YKLRP and DPYKLRP) that being among the most abundant in each fraction also fulfilled the common structural features described for many ACE-inhibitory peptides derived from food proteins.[18] Since the role of specifically C-terminal P residue in enhancing inhibition has been highlighted in most effective antihypertensive sequences derived from milk proteins,[1] the peptides PYKLRP and GILRP identified in the most active fraction (F11) were also included in the study despite not being abundant. Interestingly the yeast proteolytic system produced the set of sequences DPYKLRP, PYKLRP and YKLRP differing in the amino acidic residue at the N-terminal end. With the aim of establishing sequence-inhibitory potency relationships, the peptides KLRP and LRP were also synthesized.

Only the six peptides having a P residue at the C-terminal end showed detectable inhibitory activity at 20 μM under our *in vitro* assay conditions. Further concentration response curves allowed the determination of IC_{50} values (Table 2) which varied over a 200-fold range. The higher potency as indicated by lower IC_{50} value corresponded to the tripeptide LRP.

Table 2. Inhibitory Potency of Selected LF-Derived Peptides on ACE Activity

Peptide	IC_{50} (μM)[a]
DPYKLRP	30.5 ± 1.4 (c)
PYKLRP	10.2 ± 1.2 (b)
YKLRP	16.5 ± 0.7 (b)
KLRP	91.6 ± 4.0 (d)
LRP	0.35 ± 0.03 (a)
GILRP	90.7 ± 5.0 (d)

[a]Inhibitory potency is expressed as IC_{50} and data are mean ± SEM of at least 3 independent experiments. Data with the same letter are not significantly different, $P > 0.05$ (one way ANOVA followed by Student-Newman-Keuls test).

3.3 Antihypertensive effect of LF-derived peptides

The antihypertensive effect of the six ACE-inhibitory peptide sequences was characterized in SHRs. Average SBP, measured by the tail-cuff method in awake SHRs, was 200 ± 1 mm Hg (n = 58). Oral administration of the six LF-derived peptides at 10 mg/kg induced significant reductions in SBP as shown in Figure 1, together with the lack of effect of oral saline and the antihypertensive effect of captopril (50 mg/kg). Similar to the effect caused by captopril, the antihypertensive effect of sequences DPYKLRP, GILRP and LRP remained significant up to 24 h post administration. Antihypertensive effects ranged from -26.8 mm Hg for both DPYKLRP and LRP till -13.2 mm Hg for KLRP. Reductions in SBP caused by DPYKLRP (-26.8 ± 2.4 mm Hg; 1 h post administration) and LRP (-26.8 ± 1.3 mm Hg; 2 h) were comparable to that of the captopril control (-27.9 ± 2.1 mm Hg; 1 h) (one-way ANOVA; $P>0.05$).

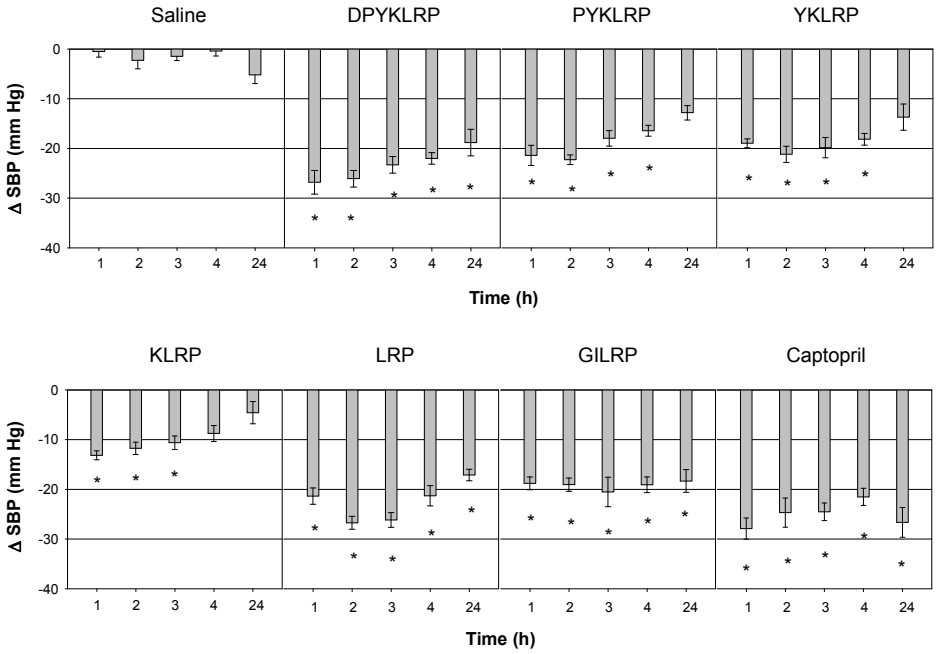

Figure 1. Time course of systolic blood pressure (SBP) changes after oral administration of physiological saline, captopril (50 mg/kg) and LF-derived peptides (10 mg/kg) to SHRs. Pressure changes (ΔSBP) are expressed in absolute values (mm Hg) and data are expressed as mean ± SEM from 6-7 determinations. **P*<0.01 versus control saline group (one-way ANOVA followed by Dunnett multiple comparison tests).

The heptapeptide DPYKLRP and the tripeptide LRP were further studied for dose-dependent antihypertensive effects. Both peptides induced significant dose-dependent (3, 7 and 10 mg/kg) reductions in SBP at each time point from 1 h to 24 h after oral administration (Figure 2).

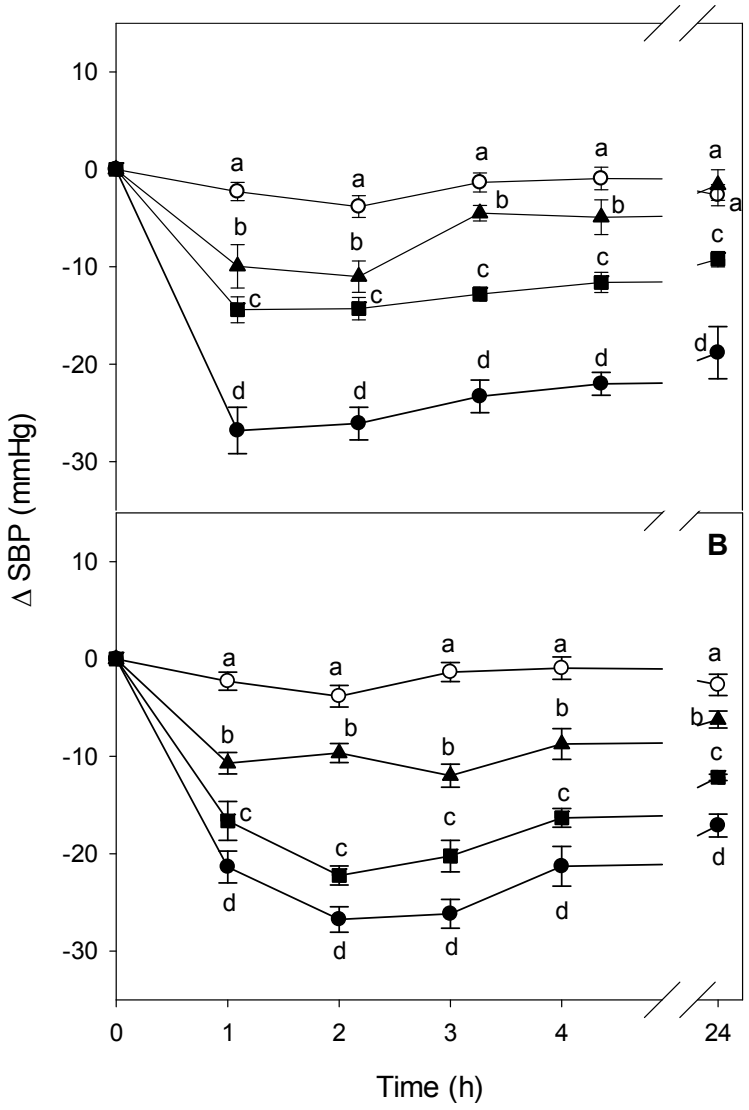

Figure 2. Time course of systolic blood pressure (SBP) changes after oral administration of increasing doses of DPYKLRP (A) and LRP (B) to SHRs. (○) physiological saline, (▲) 3 mg/kg, (■) 7 mg/kg, (●) 10 mg/kg. Pressure changes are expressed in absolute values (mm Hg) and data are expressed as mean ± SEM from 5-8 determinations. Different letters indicate significant differences among doses at each time point (one-way ANOVA followed by Student-Newman-Keuls multiple comparison tests, $P<0.05$).

3.4 Resistance of LF-derived peptides to gastrointestinal enzymes

The six antihypertensive peptides were subjected to a hydrolysis process which simulates gastrointestinal digestion due to the action of gastric and pancreatic enzymes. The analysis of digests by RP-HPLC (Figure 3) showed that the longer sequences, DPYKLRP and PYKLRP, were completely hydrolyzed releasing several fragments. A partial hydrolysis was observed for the pentapeptide YKLRP (approximately 60% of the initial concentration of the input peptide). In the conditions tested, sequences KLRP and GILRP were slightly hydrolyzed (approximately 6% and 12% decrease from the initial concentrations) whereas LRP was resistant to gastrointestinal enzymes. Noteworthy, in the gastrointestinal digests of the hydrolyzed peptides, the sequences LRP and KLRP were detected among others. LRP at concentrations of 525 μM, 600 μM and 465 μM were detected in the digests of DPYKLRP, PYKLRP and YKLRP, respectively. Also a minor quantity of LRP (3 μM) was detected in the KLRP digest. In the conditions tested, the sequence LRP was not detected in the GILRP digest. With respect to KLRP, concentrations of 550 μM and 140 μM were detected in the digests of DPYKLRP and PYKLRP. Also the sequence KLRP was detected at a concentration of 17 μM in the YKLRP digest.

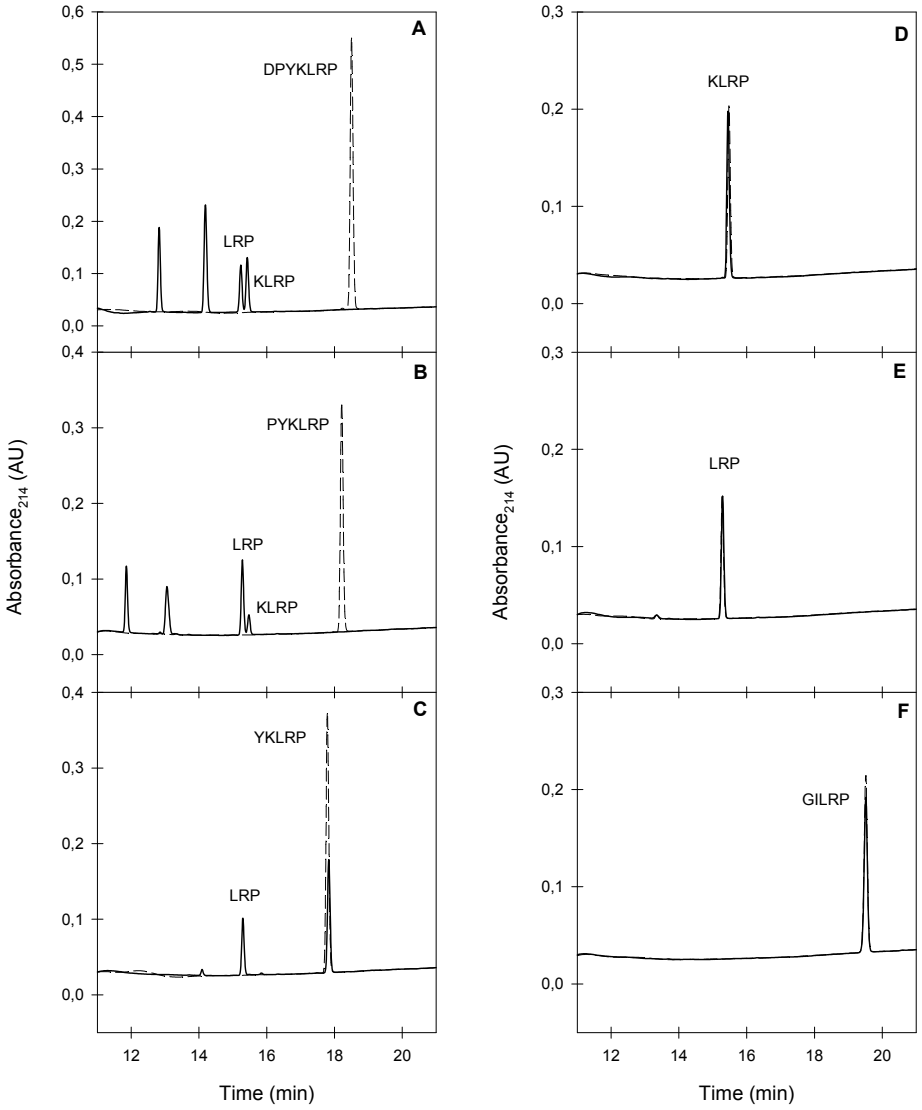

Figure 3. RP-HPLC chromatograms of peptides before (dashed line) and after (solid line) being submitted to a simulated gastrointestinal digestion. (A) DPYKLRP, (B) PYKLRP, (C) YKLRP, (D) KLRP, (E) LRP, (F) GILRP.

3.5 Effects of LF-derived peptides on blood components of the renin-angiotensin system

The effects of DPYKLRP and LRP (10 mg/kg) on serum ACE activity and angiotensin II levels, and on plasma aldosterone levels were studied in SHRs. Captopril (50 mg/kg) was also included as a positive control.

The average serum ACE activity for all measurements carried out in the three experimental groups before treatment intake was 111.4 ± 1.8 U/L (n=22). As shown in Figure 4A, ACE activity was significantly reduced in SHRs treated with DPYKLRP, LRP and captopril at 1 h and 4 h post administration, and reverted to initial values after 24 h. At 1 h post administration, when maximum effects were observed, the reduction in ACE activity induced by DPYKLRP (48.1 ± 2.5%) was similar to that caused by captopril (43.4 ± 3.1%), and significantly higher than the reduction induced by LRP (19.1 ± 2.7%) in SHRs (one way ANOVA followed by Student-Newman-Keuls test).

SHRs showed an average serum angiotensin II level of 71.2 ± 1.3 pg/mL (n=22) before treatment intake. Angiotensin II levels in SHRs were significantly reduced by the three treatments at 1 h post administration (Figure 4B). The effect of LRP reverted at 4 h post administration whereas the reductions caused by the heptapeptide and captopril reverted at 24 h. When maximum effects were observed (1 h), the effects caused by DPYKLRP (27.1 ± 0.6% reduction in angiotensin II levels) and captopril (33.2 ± 1.3%) were similar and higher than that provoked by LRP treatment to SHRs (14.8 ± 1.9%; one way ANOVA followed by Student-Newman-Keuls test).

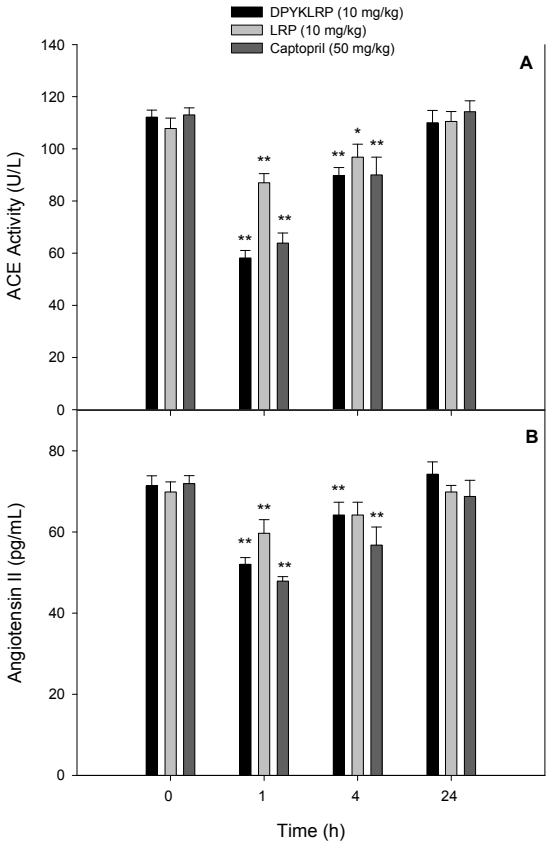

Figure 4. Time course of changes produced in the levels of blood components of the renin-angiotensin system after oral administration of DPYKLRP (10 mg/kg), LRP (10 mg/kg) and captopril (50 mg/kg) to SHRs. (A) Serum angiotensin I-converting enzyme (ACE) activity. (B) Serum angiotensin II levels. Data are mean ± SEM from 4-10 determinations. **P*<0.05 versus baseline values at time 0 h, ***P*<0.01 versus baseline values at time 0 h (one-way ANOVA followed by Dunnett multiple comparison tests).

By contrast to that observed in serum ACE activity and angiotensin II levels, plasma aldosterone level of SHRs (244.7 ± 1.9 pg/mL; n=22) was not significantly affected by any of the treatments (data not shown).

4. Discussion

Yeast products have been used for many years as ingredients and additives in food processing, although their potential bioactivity has been less investigated.[19] *K. marxianus*, considered a GRAS (Generally Recognized As Safe) microorganism, has been isolated from a great variety of habitats, which results in a high metabolic diversity. Therefore, different biotechnological applications of this yeast including production of enzymes, of single cell-protein, and of aroma compounds as well as production of bioingredients from cheese-whey have been described.[20] Moreover the beneficial properties of *K. marxianus* as a human probiotic have been recently assessed.[21]

In this study, we have identified four novel LF-derived peptides which are reported as ACE-inhibitory and antihypertensive sequences for the first time. To the best of our knowledge, DPYKLRP, PYKLRP, YKLRP and GILRP produced by the proteolytic system of *K. marxianus* Km2 strain when grown in LF as sole nitrogen source, are the first peptides with antihypertensive effects after oral administration to SHRs produced by a food-isolated yeast strain. Novel sequences identified here could at least in part contribute to the ACE inhibiting and antihypertensive effects of *K. marxianus* pLFH.[14]

The four *K. marxianus* ACE-inhibitory peptides have a C-terminal P residue. It has been described that the rigid structure of this amino acid may lock the carboxyl group into a conformation favorable for interaction with the positively charged residue at the active site of the enzyme.[22] Also the four sequences share the C-terminal tripeptide LRP. Interestingly LRP, which can be found in three different regions of LF sequence, was pointed out as the sequence responsible of the *in silico* high ACE-inhibitory activity of different peptide sequences in LF, and in accordance with our results, an

IC_{50} value of 0.27 μM was described for the tripeptide.[12] The sequence LIWKL was the most potent LF-derived peptide described so far (IC_{50} = 0.47 ± 0.01 μM).[8] Here, LRP was the most potent sequence with an IC_{50} value (IC_{50} = 0.35 ± 0.03 μM) slightly lower than that of LIWKL. Our results suggest that N-terminal elongations decrease *in vitro* inhibitory potency, although it might not result in lower antihypertensive effects (see below). Moreover elongations at the C-terminal end of the tripeptide also provoked a decrease of inhibitory potency since an IC_{50} value of 4.14 μM was described for the sequence LRPVAA.[23]

Our results in SHRs show a complex relationship between the *in vitro* ACE-inhibitory potency and the *in vivo* antihypertensive effects after oral administration suggesting a role for gastrointestinal digestion in the formation and degradation of antihypertensive peptides. When subjected to hydrolysis with gastrointestinal enzymes all of the peptides tested in this study were hydrolyzed to different degrees with the exception of LRP. Remarkably this sequence was found in most of the digests suggesting that the tripeptide might contribute to the *in vivo* effects of parental peptides. Further work will be needed to clarify the physiological relevance of LRP as well as of the other digestion fragments that could also contribute to the blood pressure-lowering effects of parental peptides.

Although the IC_{50} values of LF-derived peptides were by far higher than that of ACE-inhibitory drug captopril (0.022 μM),[24] in the conditions tested, oral administration of DPYKLRP and LRP resulted in a significant decrease in SBP (13.4% reduction from baseline) similar to that of captopril (14% reduction). These results are also in agreement with the previously reported antihypertensive effect of the LF-derived peptide LIWKL (12.1% reduction).[8] It has been reported that food-derived ACE-inhibitory peptides might possess higher *in vivo* effects than expected from *in vitro* inhibitory

potencies due to their higher affinity to target tissues and their slower elimination.[25]

It has been also postulated that other mechanisms of action apart from ACE inhibition might underlie *in vivo* antihypertensive effect of ACE-inhibitory peptides, including short-term vasoactive mechanisms as well as long term-antioxidant and anti-inflammatory mechanisms.[26] In this context the sequence GILRP isolated here is part of the sequences GILRPY and GILRPYL identified in a proteinase K LF hydrolyzate which exerted *in vivo* antihypertensive effect. Both the hydrolyzate and GILRPY showed significant endothelin converting enzyme (Capece et al.)-inhibitory effects.[9] ECE is a key peptidase in the endothelin system that cleaves precursor inactive big endothelin-1 to produce active endothelin-1 which has powerful vasoconstrictor and pressor properties.[27] The endothelin system has an increasingly recognized role in blood pressure regulation, and has also been targeted for hypertension drug treatment. Moreover, we described a set of peptides derived from LF which showed inhibitory effects on ACE and ECE activities.[28] Also the ACE-inhibitory peptide lactokinin can modulate endothelin-1 release by endothelial cells.[29] Whether the antihypertensive effect showed by GILRP in this study might be also due to ECE inhibition deserves further studies.

Dose-dependent antihypertensive effects of DPYKLRP and LRP prompted us to look for a mechanism of action responsible for the graded *in vivo* responses of the LF-derived peptides. Determinations of blood RAS components support ACE inhibition as an *in vivo* antihypertensive mechanism in SHRs. *In vivo* ACE-inhibitory effect can be assessed by measuring tissue membrane-anchored or soluble, circulating ACE activities, and confirmed by measuring circulating levels of angiotensin II.[30] Our results show that serum ACE activity is reduced in SHRs after oral administration of both peptides. Moreover, inhibition of ACE was confirmed

in peptide treated SHRs by the reduction in angiotensin II level. We have previously reported that long term administration to SHRs of an antihypertensive bovine LF pepsin hydrolyzate enriched in low molecular weight peptides reduced circulating ACE activity, angiotensin II and aldosterone levels.[10] By contrast, in the present study, the level of serum aldosterone, the adrenal endocrine component downstream angiotensin II in the renin-angiotensin axis,[2] was not affected by single-dose treatments with DPYKLRP and LRP. *In vivo* ACE inhibition has been also pointed out as the mechanism underlying the blood pressure reduction of the tripeptide IQP derived from the blue algae *Spirulina platensis* since serum ACE and angiotensin II levels were significantly reduced in SHRs after one-week treatment.[31] Nonetheless, the identification of other *in vivo* mechanisms beyond ACE inhibition underlying antihypertensive effects of the LF-derived peptides identified in this study should be further investigated.

Our results point out *K. marxianus* as a feasible GRAS microorganism for the production of novel LF-derived peptides with ACE-inhibitory and antihypertensive effects. The LF-derived peptides produced by *K. marxianus*, DPYKLRP, PYKLRP, YKLRP and GILRP, effectively decreased arterial blood pressure in SHRs and could, at least in part be responsible for the antihypertensive properties previously described for *K. marxianus* LF hydrolyzate. Also data reported here suggest ACE inhibition as *in vivo* mechanism for the antihypertensive effects of the sequences DPYKLRP and LRP in particular, although other mechanisms cannot be discarded.

5. Acknowledgement

The authors thank José Javier López-Díez and Sonia Ruiz-Piquer for technical assistance.

This work was supported by grant AGL2010-21009 from 'Ministerio de Educación y Ciencia - FEDER', Consolider Ingenio 2010, Fun-C-Food, CSD2007-00063 and RETICS INVICTUS RD12/0014/0004 from 'Instituto de Salud Carlos III'. A. García-Tejedor is recipient of a predoctoral fellowship from 'Ministerio de Educación y Ciencia' (BES-2011-044424).

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Abbreviations

ACE, angiotensin I-converting enzyme; BCA, bicinchoninic acid method; ECE, endothelin converting enzyme; LF, bovine lactoferrin; GRAS, generally recognized as safe; LAB, lactic acid bacteria; pLFH, lactoferrin permeate enriched in peptides of molecular weight lower than 3kDa; RAS, renin-angiotensin system; RP-HPLC, reversed-phase high-performance liquid chromatography; RP-HPLC-MS/MS, reversed-phase high-performance liquid chromatography tandem mass spectrometry; SBP, systolic blood pressure; SHRs, spontaneously hypertensive rats; TFA, trifluoroacetic acid.

In vivo antihypertensive mechanism of lactoferrin-derived peptides: reversion of angiotensin I- and angiotensin II-induced hypertension in Wistar rats.

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Journal of Functional Foods 15:

294-300 (2015)

ABSTRACT

Novel peptides with antihypertensive effects in SHR rats have previously been identified in lactoferrin (LF) hydrolysates. To investigate their *in vivo* antihypertensive mechanism, we have assessed the blood pressure lowering effects of two of these LF-derived peptides (RPYL and DPYKLRP) in Wistar rats subjected to either angiotensin I- or angiotensin II-induced hypertension. Blood pressure was measured by the tail-cuff method, hypertension was induced by subcutaneous infusion of angiotensins, and then captopril, valsartan or LF-derived peptides orally administered. Angiotensin I- and angiotensin II-induced hypertension were reversed by captopril and valsartan, respectively. RPYL and DPYKLRP reversed angiotensin I-induced hypertension, while DPYKLRP but not RPYL produced a modest reversion of angiotensin II-elicited hypertension. Neither RPYL nor DPYKLRP modified normotension. Thus, *in vivo* ACE inhibition is involved in the antihypertensive effects of LF-derived peptides like RPYL and DPYKLRP, while inhibition of AT_1 receptor-mediated vasoconstriction plays a less relevant role.

Keywords: antihypertensive peptides**,** lactoferrin-derived peptides**,** angiotensin-induced hypertension**,** Wistar rat**,** *in vivo* ACE inhibition**,** renin angiotensin system.

1. Introduction

Hypertension is an important modifiable risk factor for cardiovascular disease, which its management includes not only pharmacological treatment but also lifestyle changes like physical activity and dietary habits (Ruilope, 2011). The increasing perception about the relationship between food and health is fostering the development of functional foods providing health benefits beyond nutrition (Roberfroid, 2002). Some dietary proteins contain embedded peptides that once released behave as bioactive peptides with different health-promoting properties including blood pressure lowering effects (Hartmann & Meisel, 2007).

The renin angiotensin system (RAS), a key player in blood pressure and fluid balance regulation, is one of the main targets for the treatment of hypertension. Its inhibition at three possible levels, angiotensin-converting enzyme (ACE), upstream renin activity, or downstream angiotensin receptors, is the pharmacological basis for commonly used antihypertensive drugs (Fragasso et al., 2012). ACE inhibition is also the most aimed target for antihypertensive food-derived peptides developed as an alternative to drugs (Hong et al., 2008). Although different animal and plant proteins have been used, milk is the main source of antihypertensive ACE-inhibitory peptides reported to date (Hernández-Ledesma, Contreras, & Recio, 2011; Korhonen, 2009).

Despite numerous efforts, the *in vivo* mechanism underlying vasoactive and blood pressure lowering effects of antihypertensive food-derived peptides has not yet been fully established, which may hamper their use as bioactive ingredients in functional foods. A recent Scientific Opinion of the European Food Safety Authority on the substantiation of health claims related to isoleucine-proline-proline (IPP) and valine-proline-

proline (VPP) and maintenance of normal blood pressure, stated that there was no convincing evidence for a mechanism by which these widely studied bioactive peptides could exert the claimed effect (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2012). Beyond *in vivo* ACE inhibition reported for some peptides and hydrolysates (Jäkälä, Hakala , Turpeinen , Korpela, & Vapaatalo, 2009; Lu et al., 2011; Wang et al., 2012; Yang, Yang, Chen, Tzeng, & Han, 2004) antihypertensive effects could be mediated by their interaction with other RAS steps and related pathways in the vascular system, potentially contributing to blood pressure reduction (Udenigwe & Mohan, 2014).

In previous studies, antihypertensive properties of peptides derived from bovine lactoferrin (LF), a well-characterized protein of milk whey, were shown in spontaneously hypertensive rats (SHR) (Ruiz-Giménez et al., 2010). Focusing on the RAS system, we have reported *in vitro* ACE inhibition by a LF pepsin hydrolysate (named pepsin LFH <3kDa) and its antihypertensive effect in SHR rats after acute oral administration (Ruiz-Giménez et al., 2012). Moreover, chronic oral administration of pepsin LFH <3kDa to SHR rats resulted in reductions of hypertension progression, circulating ACE activity, angiotensin II and aldosterone levels, as well as a compensatory increase of renin activity (Fernández-Musoles, Manzanares, Burguete, Alborch, & Salom, 2013a). Recently, we have reported that *in vitro* inhibition of ACE activity by pepsin LFH <3kDa also occurred in cultured human endothelial cells (García-Tejedor et al., 2015). On the other hand, dairy yeasts (*Debaryomyces hansenii*, *Kluyveromyces lactis* and *K. marxianus*) produced LF-derived antihypertensive hydrolysates. Among them, the hydrolysate produced by a particular strain of *Kluyveromyces marxianus* (named Km2 pLFH) showed the highest *in vitro* ACE inhibition and *in vivo* blood pressure reduction in SHR rats (García-Tejedor, Padilla, Salom, Belloch, & Manzanares, 2013).

Novel bioactive peptides have been identified in LF hydrolysates obtained by enzymatic proteolysis or yeast fermentation, and some of them have been particularly characterized. Among peptides identified in pepsin LFH <3kDa, the tetrapeptide RPYL produced *in vitro* ACE inhibition, *ex vivo* inhibition of ACE-dependent vasoconstriction induced by angiotensin I and *in vivo* reduction of systolic blood pressure in SHR rats (Ruiz-Giménez et al., 2012). Moreover, RPYL also produced *ex vivo* inhibition of angiotensin II-elicited vasoconstriction by blocking angiotensin AT_1 receptors (Fernández-Musoles et al., 2014). On the other hand, among peptides identified in Km2 pLFH, the heptapeptide DPYKLRP produced *in vitro* ACE inhibition and *in vivo* decrease of systolic blood pressure in SHR rats. Moreover, antihypertensive effects in SHR rats were accompanied by reductions in circulating ACE activity and angiotensin II level (García-Tejedor et al., 2014). Further *in vivo* studies to elucidate the mechanism of action of milk protein-derived antihypertensive peptides are still necessary to develop dairy functional foods. In order to gain insight into the *in vivo* antihypertensive mechanism of the LF-derived peptides RPYL and DPYKLRP, we have assessed their blood pressure lowering effects in Wistar rats subjected to either angiotensin I- or angiotensin II-induced hypertension.

2. Materials and methods

2.1 Materials

Peptides (RPYL and DPYKLRP) were ordered from GenScript Corp. (Piscataway, NJ, USA) wherein they were synthesized by solid phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry. Peptide purities of supplied batches were 99.7% for RPYL and 96.9% for DPYKLRP. Angiotensin I, angiotensin II, captopril and valsartan were purchased from Sigma-Aldrich Química (Tres Cantos, Madrid, Spain). ALZET Osmotic Pumps (model 2ML4) were purchased from Charles River Laboratories (Barcelona, Spain). Diazepam and ketamine were purchased from Roche Farma (Madrid, Spain) and Parke-Davis (Alcobendas, Madrid, Spain), respectively.

2.2 Animal welfare

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 to be carried out in its accredited animal research facility.

Ten male Wistar rats (200-225 g) were supplied by Charles River Laboratories (Barcelona, Spain). Rats were housed in temperature-controlled rooms (23 °C) with 12 h light/dark cycles, and consumed tap water and standard diet *ad libitum*. A two-week period of acclimatization was allowed to recover from the stress associated with transportation (Obernier & Baldwin, 2006). To minimize the impact of light cycle and feeding on circadian rhythms of blood pressure (van den Buuse, 1999), the experiments always started at the same time in the morning (9:00 a.m.) in fasted rats.

2.3 Blood pressure measurement

Indirect measurement of systolic blood pressure (SBP) was carried out in awake restrained rats by the noninvasive tail-cuff method using computer-assisted Non-Invasive Blood Pressure equipment (LE5001 unit with LE5160R cuff and transducer, Panlab Harvard Apparatus, Cornellá, Barcelona, Spain). This method has been validated with direct intra-arterial measurements (Ibrahim, Berk, & Hughes, 2006). Before the measurements, rats were kept at 37 °C during 15 min to make the pulsations of the tail artery detectable. Each value of SBP was obtained by averaging at least three consecutive and successful measurements without disturbance of the signal. Changes in SBP were calculated as the absolute difference (in mm Hg) with respect to the basal values of measurements obtained just before starting the treatments.

2.4 Hypertension induction

Rats were anaesthetized by intraperitoneal injection of 5 mg/kg diazepam and 100 mg/kg ketamine. An ALZET Osmotic Pump (model 2ML4) was surgically implanted subcutaneously on the back, between and slightly posterior to the scapulae. The osmotic pump was filled with either angiotensin I (11.1 mg/2 mL 0.1 M acetic acid) or angiotensin II (11.1 mg/2 mL distilled water), and delivered continuously for 4 weeks at a rate of 2.5 µL/hr, that is around 1 mg angiotensin/kg/day. The SBP was measured before angiotensin infusion (zero time) and twice a week during 24 days of infusion. Physiological saline was infused as negative control.

2.5 Assay of lactoferrin-derived peptides

Peptides (RPYL or DPYKLRP, 10 mg/kg) were orally administered by gastric intubation in 650 µL of physiological saline. The SBP was measured before peptide intake (zero time) and 1.5, 3, and 24 h after intake. In assays on angiotensin I-induced hypertension captopril (10 mg/kg) served as positive control, whereas in assays on angiotensin II-induced hypertension valsartan (10 mg/kg) was the positive control. The vehicles for peptides and captopril (physiological saline) and for valsartan (dimethyl sulfoxide, DMSO) were assayed as negative controls.

2.6 Data analysis and statistics

Values are expressed as the mean ± SEM. Unpaired Student's t test was used to assess differences between two groups. Analysis of variance (ANOVA) followed by Student-Newman-Keuls test or Dunnett's test was used for multiple comparisons among more than two groups. P values <0.05 were considered significant.

3. Results

3.1 Angiotensin I and angiotensin II induced blood pressure increases

Average basal SBP in Wistar rats was 119 ± 2 mm Hg (n = 10). Continuous subcutaneous infusion of angiotensin I (1 mg/kg/day) induced increase in SBP (Figure 1), which became significantly higher than SBP in saline-infused control rats from day 3. Steady-state SBP levels were attained after 17 days (151 ± 7 mm Hg, n = 4 in angiotensin I *vs* 123 ± 1 mm Hg, n = 3 in saline control at this time point). Infusion of angiotensin II (1 mg/kg/day) induced increase in SBP (Figure 1), which became significantly higher than SBP in both saline control- and angiotensin I-infused rats from day 3. Steady-state SBP levels were also attained after 17 days (189 ± 1 mm Hg, n = 3 in angiotensin II *vs* 123 ± 1 mm Hg, n = 3 in saline control at this time point). SBP remained at hypertensive levels in both angiotensin I- and angiotensin II-infused rats until the end of infusion, at day 28, and returned to normotensive values upon osmotic pump withdrawal (126 ± 1 mm Hg, n = 4; and 123 ± 1 mmHg, n = 3, respectively).

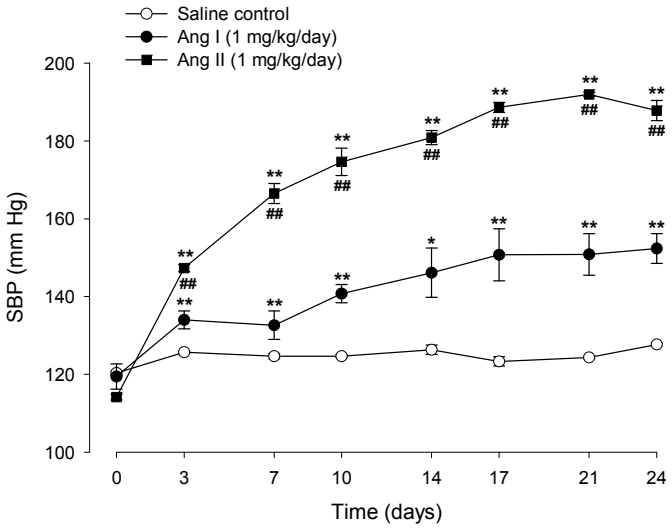

Figure 1. Time course of systolic blood pressure (SBP) during subcutaneuous continuous infusion (1 mg/kg/day) of angiotensin I and angiotensin II to Wistar rats by means of an osmotic pump. Physiological saline was infused as negative control. SBP is expressed in mm Hg and values are mean ± SEM from 3-4 determinations. *P<0.05 versus saline control, **P<0.01 versus saline control, and ##P<0.01 versus angiotensin I (one-way ANOVA followed by Student-Newman-Keuls tests).

3.2 Lactoferrin-derived peptides reversed angiotensin I-induced hypertension

In angiotensin I-induced hypertensive rats (155 ± 2 mm Hg, n = 26), oral boluses (10 mg/kg) of RPYL or to a higher extent DPYKLRP induced transient decreases in SBP. Values of SBP were maximally reduced at 1.5 h after peptide intake, remained significantly reduced at 3 h, and returned to basal values at 24 h (Figure 2). For comparison, oral captopril (10 mg/kg) elicited decrease in SBP which was maximal at 1.5 h after intake and remained significant even at 24 h (Figure 2). Decreases in SBP induced by the two peptides and captopril at 1.5 h were significantly different among

them: RPYL < DPYKLRP < captopril (P<0.05, Student-Newman-Keuls test).

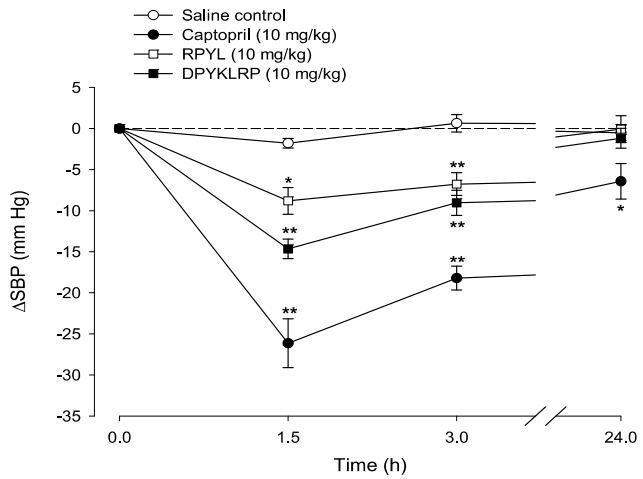

Figure 2. Time course of systolic blood pressure (SBP) after oral boluses (10 mg/kg) of lactoferrin-derived peptides RPYL and DPYKLRP administered to angiotensin I-induced hypertensive Wistar rats. Captopril was administered as positive control, whereas the vehicle for peptides and captopril (physiological saline) served as negative control. SBP change (ΔSBP) is expressed in absolute values (mm Hg) and data are mean ± SEM from 4-8 determinations. *P<0.05 versus saline control, **P<0.01 versus saline control (one-way ANOVA followed by Dunnett's tests).

3.3 Heptapeptide DPYKLRP but not tetrapeptide RPYL reversed angiotensin II-induced hypertension

With regard to angiotensin II-induced hypertensive rats (183 ± 2 mm Hg, n = 34), oral boluses (10 mg/kg) of DPYKLRP but not RPYL induced transient decrease in SBP. Values of SBP were significantly reduced only at 1.5 h after DPYKLRP intake, and returned to basal values at 24 h (Figure 3). For comparison, oral valsartan (10 mg/kg) elicited decrease in SBP (when compared to DMSO control) which was maximal at 1.5 after intake and remained significant even at 24 h (Figure 3). In contrast, captopril (10

mg/kg) did not significantly modify SBP level in angiotensin II-induced hypertensive rats (Figure 3).

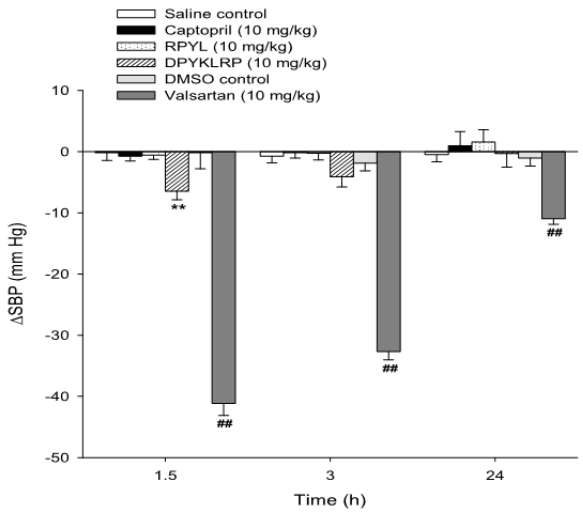

Figure 3. Time course of systolic blood pressure (SBP) after oral boluses (10 mg/kg) of lactoferrin-derived peptides RPYL and DPYKLRP administered to angiotensin II-induced hypertensive Wistar rats. Valsartan was administered as positive control, whereas captopril served to check for angiotensin converting enzyme inhibition. The vehicles for peptides and captopril (physiological saline) and for valsartan (dimethyl sulfoxide, DMSO) were administered as negative controls. SBP change (ΔSBP) is expressed in absolute values (mm Hg) and data are mean ± SEM from 4-6 determinations. **P<0.01 versus saline control (one-way ANOVA followed by Dunnett's tests), ##P<0.01 versus DMSO control (unpaired Student's t test).

3.4 Lactoferrin-derived peptides did not modify normotensive blood pressure levels

Neither lactoferrin-derived peptides (RPYL and DPYKLRP) nor drugs (captopril and valsartan), orally administered at the same dose used in hypertensive rats (10 mg/kg), produced significant changes on normortensive SBP levels in Wistar rats (Table 1).

Table 1. Time course of systolic blood pressure (SBP) in normotensive Wistar rats after oral administration of lactoferrin-derived peptides (RPYL and DPYKLRP), captopril and valsartan.

	Time (h)			
	0	1.5	3	24
Saline[a] **control**	124 ± 1 (6)	125 ± 1 (6)	126 ± 1 (6)	125 ± 2 (6)
Captopril (10 mg/kg)	127 ± 2 (5)	126 ± 2 (5)	125 ± 1 (5)	124 ± 2 (5)
RPYL (10 mg/kg)	126 ± 3 (5)	125 ± 2 (5)	129 ± 2 (5)	127 ± 3 (4)
DPYKLRP (10 mg/kg)	124 ± 2 (6)	125 ± 1 (6)	125 ± 1 (6)	126 ± 1 (6)
DMSO[b] **control**	127 ± 2 (6)	128 ± 2 (5)	127 ± 1 (5)	127 ± 2 (5)
Valsartan (10 mg/kg)	125 ± 3 (3)	127 ± 1 (3)	124 ± 2 (3)	124 ± 1 (3)

SBP is expressed in mm Hg and values are mean ± SEM from (n) determinations.
[a]Vehicle for captopril, RPYL and DPYKLRP.
[b]Dimethyl sulfoxide, vehicle for valsartan.

4. Discussion

We have found that both the tetrapeptide RPYL and to a higher extent the heptapeptide DPYKLRP reverse angiotensin I-induced hypertension when orally administered to Wistar rats. Moreover, DPYKLRP also produces a modest reversion of angiotensin II-elicited hypertension. Of note, neither RPYL nor DPYKLRP modified arterial blood pressure in normotensive rats. We and others have extensively used the SHR rat as hypertension model to assess the antihypertensive effects of food protein-derived bioactive peptides (Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012). However, this approach does not allow knowing the *in vivo* antihypertensive mechanism, unless for example blood components of the RAS are determined (Fernández-Musoles et al., 2013a; Lu et al., 2011). Alternatively, angiotensin I- or angiotensin II-induced hypertension rat models have been used to gain insight into the *in vivo* antihypertensive mechanism of diverse non-drug natural products (Liu et al., 2003; Prasad, 2013; Waghulde, Mohan, Kasture, & Balaraman, 2010). In the present study, we have combined both angiotensin I- and angiotensin II-induced hypertension in order to discriminate between effects on ACE activity and effects on downstream activation of angiotensin AT_1 receptors of LF-derived peptides.

Antihypertensive effects in SHR rats have previously been shown for both RPYL (Ruiz-Giménez et al., 2012) and DPYKLRP (García-Tejedor et al., 2014). In the present study, both RPYL and DPYKLRP reversed angiotensin I-induced hypertension in Wistar rats. Like in SHR rats, the magnitude and duration of the antihypertensive effect was higher for DPYKLRP than for RPYL on angiotensin I-induced hypertension. We used the ACE inhibitor drug captopril as a positive control, which showed antihypertensive effects. It has been previously reported that captopril, at the same dose used in our study, produces almost complete inhibition of

plasma ACE activity and attenuation of pressor responses to angiotensin I (Levens, Peach, Vaughan, Weed, & Carey, 1981). Therefore, angiotensin I-induced hypertension is a suitable model to assess *in vivo* ACE inhibition, and then our results support ACE inhibition as antihypertensive mechanism for the LF-derived peptides RPYL and DPYKLRP. Few studies have previously assessed *in vivo* ACE inhibitor effect of milk-derived peptides by means of angiotensin I administration. Milks fermented using two strains of *Lactobacillus helveticus* produced inhibition of angiotensin I-elicited acute pressor responses in anaesthetized Sprague Dawley rats (Fuglsang, Rattray, Nilsson, & Nyborg, 2003b). In contrast, milk-derived short peptides produced no effect or very moderate inhibition of these angiotensin I-elicited pressor responses (Fuglsang, Nilsson, & Nyborg, 2003a) On the other hand, a peptide concentrate obtained from hydrolysis of bovine whey brought about by *Cynara cardunculus* cardosins (PepC) and an α-lactalbumin-derived peptide identified in PepC (KGYGGVSLPEW) produced inhibition of angiotensin I-elicited acute pressor responses in anaesthetized SHR rats (Tavares, Sevilla, Montero, Carrón, & Malcata, 2012). In contrast to these previous studies in anesthetized rats, our *in vivo* assays were carried out in awake, shortly restrained rats, with a steady-state level of angiotensin I-induced hypertension, which resembles established hypertension in SHR rats.

We have previously shown that antihypertensive LF-derived peptides induce some changes in blood RAS components of SHR rats. On one hand, the pepsin LFH <3kDa hydrolysate in which RPYL was identified induced reductions of circulating ACE activity, angiotensin II and aldosterone levels, as well as a compensatory increase of renin activity (Fernández-Musoles et al., 2013a). On the other hand, DPYKLRP also induced reductions of circulating ACE activity and angiotensin II level (García-Tejedor et al., 2014). Thus, reversions of angiotensin I-induced

hypertension observed in the present study are in line with reported changes in blood RAS components, and all together consistently support ACE inhibition as *in vivo* antihypertensive mechanism for LF-derived peptides like RPYL and DPYKLRP.

As for alternative antihypertensive mechanisms beyond ACE inhibition, our prior *ex vivo* study carried out in isolated arteries showed inhibitory effects of pepsin LFH <3kDa hydrolysate and several LF-derived peptides including RPYL on angiotensin II-induced vasoconstriction because of an angiotensin AT_1 receptor blocking effect (Fernández-Musoles et al., 2014). This prompted us to assess in the present study the *in vivo* effects of the LF-derived peptides RPYL and DPYKLRP on activation of angiotensin AT_1 receptors. Our results showed that DPYKLRP produced a modest reversion of angiotensin II-elicited hypertension. We used the angiotensin AT_1 receptor antagonist drug valsartan as positive control, which showed strong antihypertensive effects, as previously reported (Kobayashi, Imanishi, & Akasaka, 2006). In contrast, the ACE inhibitor drug captopril did not modify angiotensin II-elicited hypertension, as expected (Textor, Brunner, & Gavras, 1981). Therefore, angiotensin II-induced hypertension is a proper model to assess *in vivo* inhibition of angiotensin AT_1 receptors, and then our results suggest a less relevant role for inhibitory effect on vasoactive responses mediated by angiotensin AT_1 receptors as antihypertensive mechanism for the assayed LF-derived peptides. The fact that *ex vivo* angiotensin AT_1 receptor blocking effect of RPYL was not confirmed in the present *in vivo* study points to oral peptide bioavailability and raises the question about the final active form of food derived antihypertensive peptides (Hernández-Ledesma et al., 2011). As far as we know, no study has previously assessed *in vivo* effects of milk-derived peptides on vasoactive responses mediated by angiotensin AT_1 receptors by means of angiotensin II administration.

Finally, beyond their effects on different steps of the RAS system, we have reported that some LF-derived peptides different to those studied here may act as dual vasopeptidase inhibitors since, in addition to ACE, they can also produce *in vitro* inhibition of endothelin-converting enzyme (Capece et al.) activity and *ex vivo* inhibition of ECE-dependent vasoconstriction (Fernández-Musoles et al., 2010, 2013b). Since ECE is a key enzyme of the endothelin system, which is also involved in vascular tone and blood pressure regulation, *in vivo* participation of this inhibitory mechanism in the antihypertensive effects of LF-derived peptides like RPYL and DPYKLRP deserves further research.

5. Conclusions

Reversion of angiotensin I-induced hypertension by RPYL and DPYKLRP point to *in vivo* ACE inhibition as a mechanism involved at least in part in the antihypertensive effects of these LF-derived peptides. On the other hand, slight reversion of angiotensin II-induced hypertension by DPYKLRP, and no effect at all of RPYL, suggests a less relevant role for an inhibitory effect on vasoactive responses mediated by angiotensin AT_1 receptors as antihypertensive mechanism for these bioactive peptides. Finally, it should be also noted that the effect of RPYL and DPYKLRP were specific to the angiotensin-induced hypertensive states, because these peptides did not modify the arterial blood pressure of normotensive rats. Individual peptides could be applied as nutraceuticals in health-promoting functional foods for the treatment of hypertension.

6. Acknowledgements

This work was supported by grant AGL2010-21009 from 'Ministerio de Educación y Ciencia - FEDER', Consolider Ingenio 2010, Fun-C-Food, CSD2007-00063 and RETICS INVICTUS RD12/0014/0004 from 'Instituto de Salud Carlos III'. A. García-Tejedor is recipient of a predoctoral fellowship from 'Ministerio de Educación y Ciencia' (BES-2011-044424).

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Abbreviations

ACE, angiotensin-I-converting enzyme; ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; ECE, endothelin-converting enzyme; LF, lactoferrin; RAS, renin angiotensin system; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat

An antihypertensive lactoferrin hydrolysate inhibits angiotensin I-converting enzyme, modifies expression of hypertension-related genes and enhances nitric oxide production in cultured human endothelial cells.

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Journal of Functional Foods 12:

45-54 (2015)

ABSTRACT

This study was aimed to explore whether an antihypertensive lactoferrin hydrolysate (LFH) can inhibit angiotensin I-converting enzyme (ACE) activity and modify the expression of genes related to hypertension in human umbilical vein endothelial cells (HUVEC). LFH induced significant inhibition of ACE activity but it did not affect ACE mRNA levels after 24 h of exposure. LFH treatment significantly affected the expression of genes encoding for proteins involved in nitric oxide pathway such as soluble guanylate cyclase 1 α3 subunit (GUCY1A3; 4.42-fold increase) and nitric oxide synthase trafficking (NOSTRIN; 2.45-fold decrease). Furthermore, expression of the PTGS2/COX-2 gene encoding prostaglandin-endoperoxide synthase 2 a key component of prostaglandin synthesis was significantly increased (2.23-fold). Moreover, NOSTRIN mRNA downregulation was consistent with reduced NOSTRIN protein expression and increased NO production observed in HUVEC. The present study reveals the complexity of the effects exerted by LFH opening avenues for the better understanding of its antihypertensive effects.

Keywords: lactoferrin hydrolysate, ACE inhibition, endothelial cells, transcriptomic analysis, NOSTRIN, nitric oxide.

1. Introduction

Hypertension is one of the most prevalent risk factors associated with cardiovascular diseases, the leading cause of death in Western countries. During the last two decades many studies have focused on the dietary prevention of hypertension development, with particular interest in food-derived bioactive peptides with inhibitory effects on angiotensin I converting enzyme (ACE). Some of these food-derived peptides can control arterial blood pressure in hypertensive animals after a single oral dose and also during chronic administration. Moreover, some of them may reduce systolic and diastolic blood pressure in hypertensive patients (Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012).

ACE, as part of the renin-angiotensin-aldosterone system (RAAS), hydrolyzes both the inactive angiotensin I into vasoconstrictor angiotensin II and the vasodilator bradykinin into inactive peptide fragments leading to increased blood pressure (Carey & Siragy, 2003). Most well-known effects of angiotensin II, including vasoconstriction and release of vasopressin and aldosterone, are mediated by angiotensin type 1 receptors (AT1) (Brewster & Perazella, 2004) whereas bradykinin acts mainly via kinin type 2 receptors (B2) which lead to the production of nitric oxide (NO), a vasorelaxing factor (Hillmeister & Persson, 2012).

Despite numerous efforts, the *in vivo* mechanism underlying vasoactive and blood pressure lowering effects of antihypertensive food-derived peptides has not yet been fully established. Beyond *in vivo* ACE inhibition reported for some peptides and hydrolysates (Yang, Yang, Chen, Tzeng, & Han, 2004; Lu et al., 2011; Wang et al., 2012; García-Tejedor et al., 2014), antihypertensive effects can be mediated via other components of the RAAS system, such as renin which catalyzes the cleavage of the N-terminal region of angiotensinogen to release angiotensin I (Udenigwe, Lin, Hou, & Aluko, 2009; Fitzgerald et al., 2012; Ajibola, Fashakin, Fagbemi, &

Aluko, 2013). Moreover, emerging evidence points to the arginine–nitric oxide pathway, the endothelin system or opioid receptors as molecular targets for food-derived antihypertensive peptides (Udenigwe & Mohan, 2014).

In previous studies, antihypertensive properties of peptides derived from bovine lactoferrin (LF), a well-characterized component of milk whey, were extensively characterized (Centeno et al., 2006; Ruiz-Giménez et al., 2010). Focusing on the RAAS system, we have shown vasoactive effects of a LF pepsin hydrolysate (LFH) through ACE inhibition (Ruiz-Giménez et al., 2007) and its orally antihypertensive effect in spontaneously hypertensive rats (SHR) after acute administration (Ruiz-Giménez et al., 2012). Moreover, chronic administration of LFH resulted in reductions of circulating ACE activity, angiotensin II and aldosterone levels, as well as a compensatory increase of renin activity (Fernández-Musoles, Manzanares, Burguete, Alborch, & Salom, 2013a). Recently we have suggested that inhibition of angiotensin II-induced vasoconstriction by blocking angiotensin AT1 receptors is a potential mechanism also contributing along with ACE inhibition to the antihypertensive effect of LFH (Fernández-Musoles et al., 2014). Beyond its effect on the RAAS system, LFH might act as dual vasopeptidase inhibitor since it also inhibits endothelin-converting enzyme (Capece et al.), a key enzyme of the endothelin system involved in vascular tone and blood pressure regulation (Fernández-Musoles et al., 2010; Fernández-Musoles et al., 2013b).

Endothelial cells line the internal surface of blood vessels, play critical roles in vascular biology and represent a good model to evaluate the molecular mechanisms involved in blood pressure regulation. Since the effects of antihypertensive peptides might result from synergic interactions with several targets, global approaches represent a feasible strategy for revealing the action of these peptides through distinct pathways. With the

aim of characterizing the effect of LFH at the cellular level, its inhibitory effect on ACE activity in cultured human endothelial cells was determined. Moreover, the effects of LFH on the expression of a panel of genes related to hypertension were evaluated in these cells. Finally, in the light of mRNA expression results, the effects of LFH on the NO pathway, including NO production, have been specifically assessed.

2. Materials and methods

2.1 Materials

Bovine LF was provided by FrieslandCampina Domo (Zwolle, The Netherlands). Bicinchoninic acid protein assay kit, porcine pepsin, gelatin, monoclonal anti-β-actin antibody and trypan blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). ACE substrate o-aminobenzoyl-Gly-p-nitro-Phe-Pro was provided by Bachem Feinchemikalien (Bubendorf, Switzerland). Primary human umbilical vein endothelial cells (HUVEC) pooled from two to four different umbilical cords (Advancell, Barcelona, Spain) were a kind gift of Dr. Isabel Fariñas (Department of Cellular Biology, Universidad de Valencia, Spain). EndoGRO-VEGF complete medium kit was provided by Millipore (Darmstadt, Germany). Phosphate buffered saline solution (PBS) and trypsin-EDTA solution were obtained from HyClone (Logan, UT, USA). RNeasy Mini Kit, RT^2 First Strand Kit and Human Hypertension RT² Profiler™ PCR Array from SABiosciences were purchased from Qiagen (Valencia, CA, USA). LightCycler® 480 SYBR Green I Master and Complete Mini protease-inhibitor cocktail were provided by Roche Diagnostics (Basel, Switzerland). Protran BA 85 nitrocellulose, horseradish peroxidase-linked species-specific secondary antibodies and ECL Select™ Western Blotting Detection Reagent were purchased from GE Healthcare (Buckinghamshire, United Kingdom). Polyclonal anti-mNOSTRIN antibody was a kind gift of Dr. Stephanie Oess (Goethe Universität, Frankfurt am Main, Germany).

2.2 Bovine lactoferrin enzymatic hydrolysate (LFH) and synthetic peptides

Bovine lactoferrin was hydrolyzed using porcine pepsin and the product was subjected to ultrafiltration through a polyethersulfone membrane with a 3 kDa cut-off (Vivascience, Sartorius Stedim Biotech,

Aubagne, France) as previously described (Ruiz-Giménez et al., 2012). The permeate (LFH) was kept at -20^{o}C until use.

Peptides of sequences LIWKL and RPYL were purchased at >95% purity from GenScript Corporation (Piscataway, NJ, USA), wherein they were synthesized by solid phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry. Stock solutions (10 mM) of each peptide were prepared in water, sterilized by filtration and stored at -20^{o}C.

Protein content of LFH was estimated by the bicinchoninic acid method (BCA) using bovine serum albumin as standard (Ruiz-Giménez et al., 2012). Synthetic peptide concentration was based on the dry weight of the peptides.

2.3 Cell culture

HUVEC were maintained in culture using EndoGRO-VEGF complete medium kit and seeded onto plasticware previously coated with a sterile solution containing 1% (w/v) gelatin in distilled water, for at least 90 minutes at 37ºC. Cell integrity was checked daily using an inverted phase-contrast microscope, and they were subcultured before reaching 90% confluence.

Cells from passages 3 to 5 were washed with PBS solution, detached with trypsin-EDTA solution, resuspended in complete medium and evaluated for viability by microscopic observation of cells stained with 0.2 % trypan blue. HUVEC were seeded onto gelatin-coated 6-well plates at a cellular density of 25.000 viable cells/cm^2. Twenty four hours after seeding, the corresponding treatment was added directly to the cell culture supernatant, and maintained in contact with the cells for additional 24 hours. The treatments were used at the following final concentrations: LFH 700 µg/mL; RPYL and LIWKL, 100 µM. At the end of the experiments cell

viability was determined by the trypan blue exclusion method. No loss of viability was observed after any of the treatments.

2.4 ACE activity measurements

Three different biological replicates of either the control (non-treated cells), or LFH-treated cells, were evaluated for ACE activity present in cell culture supernatants. Activity was measured in all the replicates just before treatment (zero time) and at 1, 3, 6 and 24h after addition of LFH. ACE activity was measured using the fluorescent method described by Sentandreu & Toldrá (2006) with some modifications. Fifty microliter samples were taken at every time point along the experiment, and transferred to a 96-well black plate. Two hundred microliter of the internally quenched fluorescent substrate o-aminobenzoyl-Gly-p-nitro-Phe-Pro (0.45 mM) were added and the production of the fluorescent product by ACE activity was recorded (excitation 320 nm, emission 405 nm) every 30 minutes for at least 4 hours using a Fluoroskan Ascent FL (ThemoFisher, Waltham, MA). Relative ACE activity in treated cells was referred to controls at the same time points.

2.5 RNA isolation and real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) Analysis

After the 24h treatment period, cell culture supernatants were aspired and stored at -20^{o}C for nitric oxide measurements. The cells were washed with PBS and lysed for obtaining total RNA and total protein extracts using RNeasy Mini Kit. Total RNA was DNase-digested on column and was finally eluted in RNase-free water. The RNA concentration and purity was measured using a Nanodrop ND1000 spectrophotometer (ThermoFisher).

All the PCR reactions were carried out in 96-well plate format in a LightCycler 480 instrument (Roche). For the transcriptomic analysis of HUVEC, 2 µg of total RNA was retro-transcribed using RT^2 First Strand Kit, which includes an additional step for genomic DNA elimination. The cDNA obtained was used as template for the quantitation of the mRNA levels of 84 genes related to hypertension, by the use of the Human Hypertension RT² Profiler™ PCR Array. The complete gene list, including housekeeping genes and PCR controls is accessible online (www.SABiosciences.com). The qPCR reactions were carried out using the RT²SYBR green qPCR mastermix. The C_T data were obtained by the LighCycler 480 software (release 1.5.0) and were analyzed by the $\Delta\Delta C_T$ method using web-based software specific for the analysis of PCR array data (SABiosciences, Qiagen). Four different biological replicates for each control or treatment condition were assayed for mRNAs quantitation.

Further quantification of mRNA levels was carried out for those genes whose expression ratio obtained using the PCR array was modified with statistical significance by a factor of 2 or greater. For that purpose, specific oligonucleotide pairs used as PCR primers were selected for guanylate cyclase 1, soluble, alpha 3 (GUCY1A3; forward: 5'-GTCCTGCAGTGTACCACGAA-3', reverse: 5'-GGCAGATTCCGGGGATTTCA-3'; product length: 140 bp), nitric oxide synthase trafficking (NOSTRIN; forward: 5'-CTCAACCCAGCCATCCTTGT-3', reverse: 5'-GATTGCTCTGCCCACCAGAA-3'; product length: 147 bp), and prostaglandin-endoperoxide synthase 2 (PTGS2; forward: 5'-CGACTCCCTTGGGTGTCAAA-3', reverse: 5'-AAGTGCTGGGCAAAGAATGC-3'; product length: 136 bp); and also for the housekeeping genes selected, namely β-actin (ACTB; forward: 5'-AGCGAGCATCCCCCAAAGTTCAC-3', reverse: 5'-GGGTGGCTTTTAGGATGGCAAGG-3'; product length: 127 bp), and ribosomal protein L13a (RPL13A; forward: 5'-

TGCCCCACAAAACCAAGCGAGG-3', reverse: 5'-AGGCTTCAGACGCACGACCTTG-3'; product length: 131 bp). In those cases, 3.5 µg of total RNA was retro-transcribed using RT^2 First Strand Kit in a final volume of 111 µL. Two microliters of that dilution was used as template in a 10 µL PCR reaction using the LightCycler® 480 SYBR green I master. Primers were used at a final concentration of 250 nM and efficiency was determined for every primer pair at this final concentration using a pooled cDNA as template. The C_T data were obtained by the LighCycler® 480 software (release 1.5.0) and were analyzed by the $\Delta\Delta C_T$ method using the Relative Expression Software Tool version 2.0.13 (Qiagen). Three different biological replicates for each control or treatment condition were assayed for specific mRNAs quantitation.

2.6 Quantitation of NOSTRIN protein levels by western blot

Flow-through from the RNA-purification column was precipitated with 4 volumes of cold acetone, let stay on ice for 30 min and centrifuged at maximum speed in a microcentrifuge for 10 min. The pellet was resuspended in buffer (50 mM Tris, 10 mM NaCl, 2 mM EDTA, 1% v/v Igepal CA 630, 5% v/v glycerol, 1mM phenylmethylsulfonyl fluoride) containing Complete Mini protease-inhibitor cocktail. Equivalent amounts of the total protein extracts were separated by SDS-PAGE and blotted onto Protran BA 85 nitrocellulose. The membranes were probed using polyclonal anti-mNOSTRIN antibody which recognizes human NOSTRIN with an apparent molecular mass slightly under 60 kDa and also using monoclonal anti-β-Actin antibody (Sigma-Aldrich). Horseradish peroxidase-linked species-specific were used as secondary antibodies. The probed membranes were developed by chemiluminiscence with ECL Select™ Western Blotting Detection Reagent using a LAS-1000 instrument (Fujifilm,

Tokyo, Japan). Densitometric analysis of the bands was performed using ImageJ 1.48v software (NIH, Bethesda, MD, USA).

2.7 Nitric oxide content

Nitric oxide generation was estimated in cell culture supernatants by quantitation of its stable product nitrite using colorimetric determination with the Griess reagent (Tsikas, 2007). In brief, cell culture supernatants were deproteinized with zinc sulphate (final concentration 1.5% w/v; 5 min on ice), centrifuged at maximum speed for 15 minutes in a microcentrifuge, and equal volumes of the clear supernatant and of the Griess reagent incubated at room temperature for 15 minutes. Absorbance at 540 nm was recorded for blanks and samples. A standard curve for nitrite was obtained using concentrations between 0 and 1.25 μM of sodium nitrite dissolved in complete HUVEC medium.

2.8 Statistics

Data are mean ± SEM of n independent assays. Significant differences between non-treated and treated HUVEC were evaluated by Student's t-test. Differences with *P*-values < 0.05 were considered significant. Data statistical analysis was performed using the GraphPad Prism 4 software (GraphPad Software Inc, La Jolla, CA, USA).

3. Results

3.1 Effect of LFH on ACE activity

In *in vitro* tests carried out with purified porcine ACE, LFH showed inhibitory potency with an IC_{50} value of 14.3 ± 3.3 μg/mL (Ruiz-Giménez et al., 2012) while in *in vivo* studies LFH provoked reduction of serum ACE activity after long-term administration to SHR (Fernández-Musoles et al., 2013a). Since variations in the inhibition profiles of ACE from different species have been reported (Vazeux, Cotton, Cuniasse, & Dive, 2001) and with the aim to further characterize LFH inhibitory effects on ACE activity at cellular level, here we have investigated such effects in cultured HUVEC which produce and secrete ACE.

Table 1 shows ACE residual activity in HUVEC incubated for 24 h with LFH (700 μg/mL) with respect to non-treated cells (control). LFH induced a significant inhibition of ACE activity. After 1 h of treatment, when maximum inhibitory effect was observed, ACE residual activity in LFH-treated cells was 15.3 ± 0.4 %.The inhibitory effect remained significant up to 24 h ($P < 0.05$; Student's *t* test) and it was higher than 60 %.

Table 1. Time-course of ACE inhibitory activity of LFH[a] in cultured human endothelial cells.

Time (h)	ACE residual activity (%)[b]
1	15.3 ± 0.4**
3	23.1 ± 0.1**
6	28.5 ± 0.8**
24	38.6 ± 0.4**

[a]Final concentration in the assay 700 μg/mL
[b]Data are expressed as the percentage of ACE residual activity with respect to a non-treated control (100%) and are the mean ± SEM of 3 independent experiments. **Significant inhibition with respect to control, $P < 0.01$ (Student's *t*-test on un-shown absolute values of ACE activity).

3.2 Effect of LFH on gene expression profile of hypertension-related genes

RT-qPCR analysis was performed to quantify relative mRNA levels of hypertension related-genes in HUVEC exposed to 700 µg/mL LFH. Gene expression in HUVEC treated with LFH was compared with expression in non-treated cells (Table 2).

Table 2. Effect of LFH[a] on hypertension-related gene expression in cultured human endothelial cells.

Gene	Description	Fold change[b]
ACE	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	-1.65
ACTA2	Actin, alpha 2, smooth muscle, aorta	-1.20
ADM	Adrenomedullin	1.58
ADRA1D	Adrenergic, alpha-1D-, receptor	1.64
ADRB1	Adrenergic, beta-1-, receptor	1.05
ARG2	Arginase, type II	**1.30***
ATP2C1	ATPase, Ca++ transporting, type 2C, member 1	1.05
ATP6AP2	ATPase, H+ transporting, lysosomal accessory protein 2	**-1.35***
AVPR1B	Arginine vasopressin receptor 1B	1.35
BMPR2	Bone morphogenetic protein receptor, type II (serine/threonine kinase)	-1.27
CAV1	Caveolin 1, caveolae protein, 22kDa	1.04
CHRNA1	Cholinergic receptor, nicotinic, alpha 1 (muscle)	1.08
CHRNB1	Cholinergic receptor, nicotinic, beta 1 (muscle)	1.08
CLIC1	Chloride intracellular channel 1	-1.33
CLIC4	Chloride intracellular channel 4	-1.15
CLIC5	Chloride intracellular channel 5	-2.70
ECE1	Endothelin converting enzyme 1	-1.18
EDN1	Endothelin 1	1.57
EDN2	Endothelin 2	-2.56
EDNRA	Endothelin receptor type A	-1.23
EDNRB	Endothelin receptor type B	-1.00
EPHX2	Epoxide hydrolase 2, cytoplasmic	-1.52
GCH1	GTP cyclohydrolase 1	-1.07
GCHFR	GTP cyclohydrolase I feedback regulator	-1.43
GUCY1A3	Guanylate cyclase 1, soluble, alpha 3	**4.42****
GUCY1B3	Guanylate cyclase 1, soluble, beta 3	**1.81***
HIF1A	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	-1.13
ITPR1	Inositol 1,4,5-trisphosphate receptor, type 1	-1.09
ITPR2	Inositol 1,4,5-trisphosphate receptor, type 2	-1.10

KCNMA1	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	-1.17
MYLK	Myosin light chain kinase	1.21
MYLK2	Myosin light chain kinase 2	-1.19
NOS3	Nitric oxide synthase 3 (endothelial cell)	1.17
NOSIP	Nitric oxide synthase interacting protein	1.20
NOSTRIN	Nitric oxide synthase trafficking	**-2.45****
NPR1	Natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A)	-1.56
P2RX4	Purinergic receptor P2X, ligand-gated ion channel, 4	1.22
PDE3A	Phosphodiesterase 3A, cGMP-inhibited	-1.20
PDE3B	Phosphodiesterase 3B, cGMP-inhibited	-1.27
PDE5A	Phosphodiesterase 5A, cGMP-specific	1.11
PLCG1	Phospholipase C, gamma 1	-1.31
PLCG2	Phospholipase C, gamma 2 (phosphatidylinositol-specific)	-1.13
PRKG1	Protein kinase, cGMP-dependent, type I	1.03
PTGIR	Prostaglandin I2 (prostacyclin) receptor (Nurminen et al.)	1.22
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	1.28
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	**2.23***
S1PR1	Sphingosine-1-phosphate receptor 1	-1.19
SCNN1A	Sodium channel, nonvoltage-gated 1 alpha	-1.79
SLC7A1	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	1.04
SPHK1	Sphingosine kinase 1	-1.39
SPHK2	Sphingosine kinase 2	-1.03

[a]Final concentration in the assay 700 µg/mL.
[b]Fold change was calculated by the ΔΔCT method .
*Significantly different from control $P < 0.05$;
**Significantly different from control $P < 0.01$ (Student's *t*-test; n=4).

Out of the 84 genes included in the array, expression of 51 genes could be detected in the conditions tested. LFH treatment caused statistically significant upregulation of 4 genes (ARG2, GUCY1A3, GUCY1B3 and PTGS2) and downregulation of 2 genes (ATP6AP2 and NOSTRIN). Genes whose expression was modified by a factor of 2 or greater, were GUCY1A3 (4.42-fold) and PTGS2 (2.23-fold) that were upregulated, and NOSTRIN (2.45-fold) that was downregulated. GUCY1A3 which encodes guanylate cyclase 1 α3 subunit and NOSTRIN which encodes NO synthase trafficking are related to NO generation, whereas PTGS2 (also called COX2) which encodes prostaglandin-endoperoxide synthase 2 or cyclooxygenase 2 is a key component of the prostaglandin synthesis. Based on their expression profiles, these three genes were considered for further studies.

3.3 Effect of LFH and lactoferrin-derived peptides on GUCY1A3, PTGS2 and NOSTRIN expression

LFH is a complex lactoferrin hydrolysate enriched in peptides of molecular weight lower than 3 kDa. In previous studies, we further characterized LFH by chromatographic fractionation and 38 peptides contained in the ACE-inhibitory fractions were identified. Three of the most abundant peptides corresponded to sequences LIWKL, RPYL and LNNSRAP, which showed inhibitory effects on ACE and acute antihypertensive effects in SHRs (Ruiz-Giménez et al., 2012). To determine if the two most potent antihypertensive peptides, corresponding to sequences LIWKL and RPYL, could at least in part contribute to the effect of LFH on gene expression in HUVEC, the effect of both sequences on GUCY1A3, PTGS2 and NOSTRIN relative mRNA levels was studied in independent RT-qPCR experiments using primers different to those

included in the array. For proper comparison, the effect of LFH was re-evaluated using the same self-designed primer pairs.

The effects of LFH and peptide treatments on relative mRNA levels are shown in Figure 1. Effects of LFH treatment on the expression of the three selected genes were similar to those detected by the array (Table 2), thus confirming array expression results. Regarding sequences LIWKL and RPYL, treatments (100 μM) did not change mRNA expression of any of the three genes evaluated suggesting that, in the conditions tested, these two sequences might not be responsible of the effects on mRNA levels observed after LFH treatment of HUVEC.

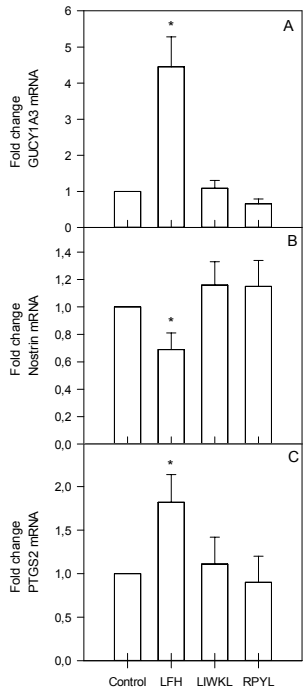

Figure 1. Effect of LFH (700 μg/mL), LIWKL (100 μM) and RPYL (100 μM) on GUCY1A3 (A), NOSTRIN (B) and PTGS2 (C) mRNA level in cultured human endothelial cells determined by qRT-PCR. Data are mean ± SEM of three independent assays. *Significantly different from control $P < 0.05$ (Student's *t* test).

3.4 Effect of LFH on NOSTRIN protein levels and on NO production

NOSTRIN is a regulatory protein which modulates NO production (Zimmermann et al., 2002), and NO-mediated vasodilation has been attributed to some food-derived peptides (Boelsma & Kloek, 2009; García-Redondo, Roque, Miguel, López-Fandiño, & Salaices, 2010). This prompted us to further study NOSTRIN as a target for LFH and to investigate whether the reduction of NOSTRIN mRNA levels detected after LFH treatment resulted in any change in protein expression and in NO production.

NOSTRIN protein levels were detected by immunoblot analysis, as can be seen in Figure 2. Twenty-four-hour treatment with LFH reduced NOSTRIN protein level by 60 % in HUVEC ($P < 0.05$; Student's *t* test). To determine the time-dependent generation of NO, measured as nitrite, HUVEC were cultured in the presence of 700 µg/mL LFH up to 24 h. Figure 3 shows enhanced nitrite production in LFH-treated HUVEC. Although this trend was observed along the time course experiment, it reached statistical significance at 24h ($P < 0.05$; Student's *t* test). At this time point, nitrite accumulated in LFH-treated HUVEC supernatants was 45% higher than that of control.

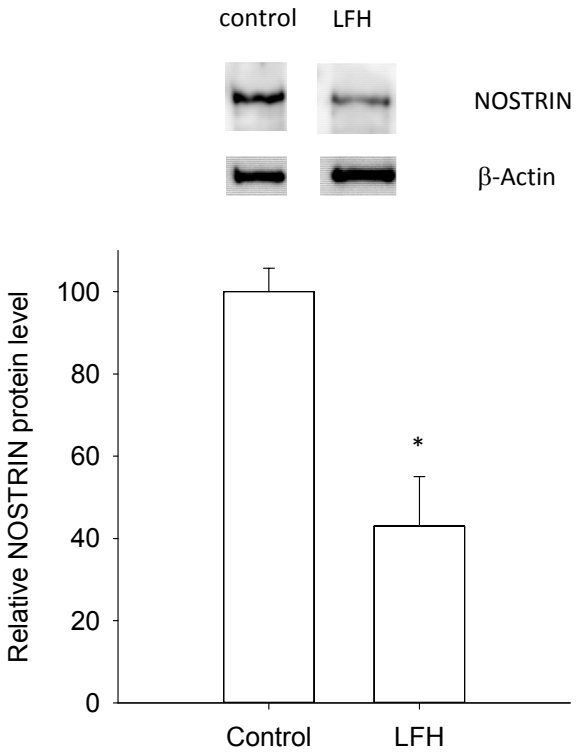

Figure 2. NOSTRIN protein level in LFH-treated cultured human endothelial cells by Western-blot analysis. Data are mean ± SEM of three independent assays. *Significantly different from control $P < 0.05$ (Student's *t*-test). A representative Western blot analysis is shown in the upper panel.

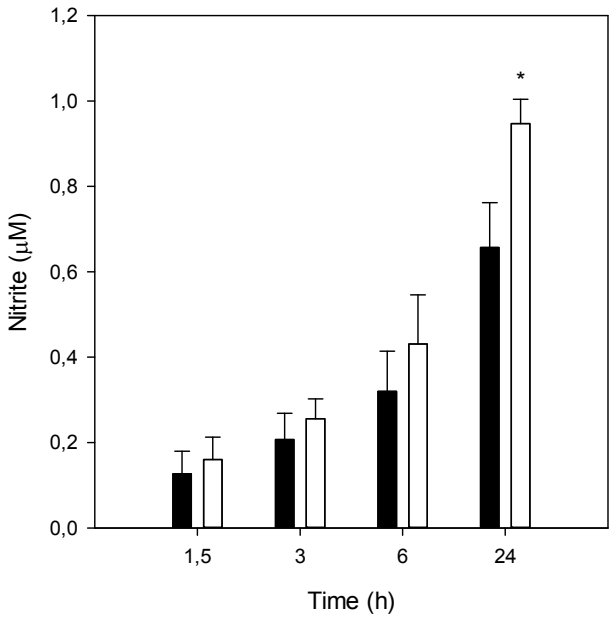

Figure 3. Time-dependent induction of NO production, measured as nitrite, by LFH in cultured human endothelial cells. Black bar: untreated-HUVEC; white bar: LFH-treated HUVEC. Data are mean ± SEM of 6 independent assays. *Significantly different from control $P < 0.05$ (Student's *t*-test).

4. Discussion

The mechanisms underlying the antihypertensive effects of food-protein derived enzymatic hydrolysates and peptides have not been yet fully established and based on recent knowledge it seems clear that multiple mechanisms besides ACE inhibition might be involved (Udenigwe & Mohan, 2014; Marques et al., 2012). In this study, we examined whether human ACE is inhibited by a complex LFH and we explored potential additional targets contributing to its antihypertensive effect by analyzing hypertension-related gene expression using cultured human endothelial cells.

Inhibition of ACE activity by LFH at a concentration of 700 μg/mL was shown in cultured HUVEC supernatants. LFH-inhibitory action on ACE was comparable to that reported for two casein trypsin hydrolysates which showed IC_{50} values in the μg/mL level (Rousseau-Ralliard et al., 2010). Despite inhibition of ACE activity at 24 h, LFH did not affect ACE mRNA levels at the same time point. Although transcriptional regulation of ACE at earlier time points cannot be discarded, our results are in agreement with previous works that describe no significant changes in expression of genes associated with RAAS in aorta of SHR after repeated administration of the well-known ACE-inhibitory peptides VPP and IPP (Yamaguchi, Kawaguchi, & Yamamoto, 2009). In HUVEC, VPP and IPP had no effect on ACE protein expression, although ACE mRNA levels were not evaluated (Hirota et al., 2011). By contrast, downregulation of the mRNA levels of renin, ACE and AT1 was reported in kidney from SHRs after repeated treatment with the ACE-inhibitory sequences IQP and RVPSL (Lu et al., 2011; Yu, Yin, Zhao, Chen, & Liu, 2014). Despite ECE has also been described as a target for LFH (Fernández-Musoles et al., 2013b), and similarly to what we describe here for ACE gene expression, we did not find significant changes in ECE relative mRNA level or in expression of other genes associated with

the endothelin system (EDN1, EDN2, EDNRA, EDNRB, see Table 2). Time-course of LFH on mRNA levels of genes associated with RAAS and endothelin system are necessary to discard any regulation at the mRNA level at shorter exposure times. Regulation via post-transcriptional or post-translational mechanisms such as mRNA stability and translation and protein-protein interactions cannot be ruled out (Chattopadhyay et al., 2005; Kohlstedt et al., 2013).

In this study, expression of the PTGS2/COX-2 gene was significantly increased by LFH treatment. COX-1 and COX-2 are the first and rate-limiting enzymes involved in the conversion of arachidonic acid to prostaglandins, thromboxane A2 and prostacyclin. It is generally assumed that COX-2 plays a detrimental role in cardiovascular homeostasis since COX-2-derived thromboxanes might induce vasoconstriction and potentiate an inflammatory state. However, in healthy humans, COX-2 generates mainly prostacyclin, a potent vasodilator and platelet inhibitor (McAdam et al., 1999). Moreover, it has been described that ACE inhibitors increase expression of COX-2 and prostacyclin levels in different experimental models (Kohlstedt, Busse, & Fleming, 2005) thus suggesting that COX-2 induction, like that provoked by LFH, may potentiate vasodilator activity. VPP and IPP treatment in SHR significantly increased the expression of COX-1 gene in aorta, but not that of COX-2 (Yamaguchi, Kawaguchi, & Yamamoto, 2009). In this work, although a slight increase in the expression of COX-1 gene was detected (1.28-fold), it did not reach statistical significance (P = 0.0609). Involvement of COX in the antihypertensive effect of milk-derived peptides deserves further research.

ACE inhibitors are considered to act protectively on endothelium by inhibiting production of angiotensin II and degradation of bradykinin, which facilitates NO production. NO is an endogenous vasodilatory gaseous molecule that continuously regulates the diameter of blood vessels and

maintains an anti-proliferative and anti-apoptopic environment in the vessel wall, thus contributing to protect the endothelium (Sessa, 2004). The action of NO as a vasodilator is mediated by the activation of vascular smooth muscle soluble guanylate cyclase (sGC), the physiological receptor for NO (Arnold, Mittal, Katsuki, & Murad, 1977) and presumably the most relevant molecular target for NO-releasing drugs in human cardiovascular therapy (Zabel, Weeger, La, & Schmidt, 1998). sGC is a signal transduction enzyme that forms the second messenger molecule cyclic GMP which in turn modulates the activity of several effector proteins that lead to vasorelaxation (Schmidt, Lohmann, & Walter, 1993). Our results showed increased NO production and upregulated expression of sGC (GUCY1) following LFH treatment in HUVEC, suggesting an elevation of cyclic GMP in HUVEC, as described for vasodilator compounds which *in vivo* activate sGC in vascular tissue (Galle et al., 1999).

Endothelial NO is produced by eNOS which converts the amino acid L-arginine into L-citrulline and NO (Geller & Billiar, 1998). eNOS is tightly controlled by co- and post-translational lipid modifications, phosphorylation on multiple residues and regulated protein-protein interactions (Qian & Fulton, 2013). Significant increases of eNOS at mRNA or protein levels in aorta of SHR after administration of VPP and IPP (Yamaguchi, Kawaguchi, & Yamamoto, 2009) or a casein hydrolysate (Sánchez et al., 2011) have been reported, as well as an increase in eNOS phosphorylation in endothelial cells cultured with ACE-inhibitory peptides (Shimizu et al., 2010; Ko et al., 2012). In our study, LFH treatment showed no effect on eNOS mRNA (Table 2) or protein levels (results not shown). By contrast, data reported here reveal eNOS-interacting protein NOSTRIN as a target for LFH in endothelial cells. In HUVEC, NOSTRIN expression was significantly downregulated after LFH treatment for 24 h. Moreover, this result was consistent with the reduced protein expression detected by immunoblot analysis (60%) and the increased NO production (45%) observed in

HUVEC after 24h LFH treatment. NOSTRIN is a protein which modulates subcellular distribution of eNOS and thus NO release. Overexpression of NOSTRIN can promote the translocation of eNOS from the plasma membrane to intracellular vesicles, with a concomitant reduction in eNOS enzyme activity and inhibition of NO synthesis (Zimmermann et al., 2002). Conversely, decreased NOSTRIN expression also influences eNOS subcellular localization and contributes to increase NO levels in endothelial cells (McCormick et al., 2011). To the best of our knowledge this is the first time that NOSTRIN is pointed out as a target for antihypertensive peptides.

Other enzymes involved in the NO pathway have been proposed as molecular targets for antihypertensive peptides. Treatment of endothelial cells with a snake venom antihypertensive peptide was related to the activation of argininosuccinate synthetase, a key enzyme of the arginine-citrulline cycle which provides arginine to eNOS (Guerreiro et al., 2009). In this context, our results show a modest but significant 30% increase in relative abundance of ARG2 mRNA in LFH-treated HUVEC. ARG2 encodes for one of the two isoforms of mammalian arginase, the enzyme which shares and competes for the substrate L-arginine with eNOS in endothelial cells. Increased expression of arginase II encoded by ARG2 reduces basal NO synthesis (Li et al., 2001), and this has been proposed as a protective mechanism against the toxicity generated by NO overproduction and NO-derived radicals (Dawson, Dawson, London, Bredt, & Snyder, 1991; Gotoh & Mori, 1999). Further research is in progress to investigate the participation of eNOS and enzymes involved in arginine metabolism in the antihypertensive effect of LFH.

In contrast to that found for LFH, the sequences LIWKL and RPYL did not elicit any significant change in the level of expression of PTGS2/COX-2, GUCY1A3 or NOSTRIN genes. These results might reveal a negligible contribution of both peptides to the effects on mRNA levels

provoked by LFH treatment, at least at the time point tested, and suggest that other peptides of the hydrolysate could contribute to such effect on gene expression in HUVEC. Synergistic effects of individual peptides cannot be discarded as suggested for the stimulatory effect on mucin gene expression in human intestinal cells of a casein hydrolysate and derived peptides (Martínez-Maqueda, Miralles, Cruz-Huerta, & Recio, 2013). Finally, stability of LFH and peptides in HUVEC during treatment has not been addressed here, and potential degradation by the action of cellular peptidases including ACE should be considered. Undoubtedly the possible effects of individual sequences contained in LFH as well as bioavailability issues merit further research.

5. Conclusions

This *in vitro* study showed that LFH was able to inhibit ACE, modify the expression of hypertension-related genes and increase NO production in human endothelial cells. The HUVEC *in vitro* model used in this study is a widely validated model and has allowed us the detailed analysis of human gene expression, although data on the mechanism of action of LFH and derived peptides in human subjects need to be obtained. Work is underway to fulfill this issue. Data from the human *in vitro* model suggest NOSTRIN as a target for LFH. NOSTRIN downregulation at both mRNA and protein levels could be partly responsible for the enhanced NO production in HUVEC at the concentration tested. Other concentrations which would be closer to a physiological situation need to be tested in order to confirm the role of NOSTRIN as a functional target of LFH. Results from the PCR array serve as a ground point for future studies on the complex mechanism of action underlying the antihypertensive effects of ACE-inhibitory peptides derived from food proteins, including lactoferrin-derived peptides.

6. Acknowledgements

This work was supported by grant AGL2010-21009 from 'Ministerio de Educación y Ciencia - FEDER', Consolider Ingenio 2010, Fun-C-Food, CSD2007-00063 and RETICS INVICTUS RD12/0014/0004 from 'Instituto de Salud Carlos III'. A. García-Tejedor is recipient of a predoctoral fellowship from 'Ministerio de Educación y Ciencia' (BES-2011-044424). Authors thank Dr I. Fariñas for kind gift of HUVEC and Dr. S. Oess for kindly providing polyclonal anti-mNOSTRIN antibody.

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Abbreviations

ACE, angiotensin I-converting enzyme; ACTB, β-actin; ARG2, arginase type II; AT1, angiotensin type 1 receptor; B2, kinin type 2 receptor; BCA, bicinchoninic acid method; ECE, endothelin converting enzyme; eNOS, endothelial nitric oxide synthase; Fmoc, N-(9-fluorenyl) methoxycarbonyl; GUCY1A3, guanylate cyclase 1, soluble, alpha 3; HUVEC, human umbilical vein endothelial cells; LF, bovine lactoferrin; LFH, lactoferrin pepsin hydrolysate; NO, nitric oxide; NOSTRIN; nitric oxide synthase trafficking; PBS, phosphate buffered saline solution; PTGS2, prostaglandin-endoperoxide synthase 2; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RASS, renin-angiotensin-aldosterone system; RPL13A, ribosomal protein L13a; SBP, systolic blood pressure; SHRs, spontaneously hypertensive rats.

Discusión general

En esta tesis doctoral se ha evaluado el potencial de levaduras no convencionales para producir hidrolizados y péptidos antihipertensivos a partir de dos proteínas lácteas, caseína (CN) y lactoferrina (LF). En función de la proteína utilizada se han utilizado distintas estrategias y aproximaciones experimentales.

En cuanto a la CN, y dado que existe mucha información sobre péptidos antihipertensivos derivados de esta proteína, nuestro objetivo fue evaluar si las levaduras eran capaces de producir secuencias peptídicas concretas (IPP, VPP, RYLGY, AYFYPEL, LHLPLP y HLPLP), cuyo potencial antihipertensivo estaba ampliamente contrastado.

Por lo que respecta a la LF, nuestro objetivo se centró en identificar nuevas secuencias peptídicas producidas por las levaduras, caracterizar su efecto antihipertensivo y profundizar en su mecanismo de acción. Para ello, se seleccionaron por su mayor potencia inhibitoria *in vitro* de la enzima conversora de angiotensina (ECA) tres hidrolizados producidos por las levaduras *D. hansenii* Dh4, *K. lactis* Kl3 y *K. marxianus* Km2. Tras demostrarse su efecto antihipertensivo en ratas espontáneamente hipertensas (SHRs), se identificaron mediante espectrometría de masas en tándem (HPLC-MS/MS) los péptidos mayoritarios presentes en el hidrolizado que mostró los efectos más potentes *in vivo,* concretamente el hidrolizado producido con la cepa de *K. marxianus* Km2. La selección de péptidos, basada en la abundancia y en la secuencia del extremo C-terminal, permitió identificar péptidos con potencia inhibitoria *in vitro* y efecto antihipertensivo en SHRs, que podrían ser responsables, al menos en parte, del efecto antihipertensivo provocado por el hidrolizado. Todas las secuencias activas presentaron un residuo de Pro en su extremo C-terminal. Además, como el sistema proteolítico de *K. marxianus* produjo las secuencias DPYKLRP, PYKLRP e YKLRP, que solamente difieren en el extremo N-terminal, se incluyeron en la caracterización los péptidos KLRP

y LRP y así se pudieron establecer relaciones entre la potencia inhibitoria y la secuencia. La caracterización de estos péptidos también incluyó ensayos de dosis-respuesta en SHRs y estudios de simulación gastrointestinal que pudieran explicar la falta de correlación entre los valores de inhibición de la ECA *in vitro* y los efectos antihipertensivos.

Además, se profundizó en el mecanismo de acción de diferentes hidrolizados y péptidos provenientes de LF. Para ello, se siguieron tres estrategias. Dos de estas estrategias se diseñaron para demostrar la inhibición *in vivo* de la ECA: por un lado, se estudiaron los efectos de los péptidos sobre la actividad de la ECA en suero de SHRs, así como sobre los niveles de angiotensina II (Ang II) y aldosterona, componentes claves del sistema renina-angiotensina-aldosterona (SRAA). Por otro lado, se indujo la hipertensión en ratas normotensas mediante la administración de angiotensina I (Ang I), lo que permitió demostrar el efecto de los péptidos sobre la hipertensión dependiente de la ECA. Asimismo, también se evaluó su efecto sobre la hipertensión inducida con Ang II, lo que permitió evaluar su interacción con los receptores AT_1. En la tercera estrategia, se utilizó un modelo diferente, en concreto células endoteliales de la vena de cordón umbilical humano (HUVEC) donde se realizaron estudios de expresión génica. Mediante matrices de PCR cuantitativa dirigidas a genes de vías de señalización relacionadas con la regulación de la presión arterial (PA) se determinó el efecto sobre la expresión génica de un hidrolizado enzimático de LF producido con pepsina.

1. Las levaduras no convencionales generan péptidos antihipertensivos a partir de proteínas lácteas

Los péptidos antihipertensivos integrados en la secuencia de las proteínas alimentarias pueden ser liberados, además de por el proceso digestivo natural, mediante la acción de enzimas o microorganismos proteolíticos (Majumder y Wu, 2014). Hasta la fecha, la producción de hidrolizados y péptidos antihipertensivos empleando microorganismos proteolíticos se había centrado en el empleo de bacterias ácido lácticas (BAL) pertenecientes a los géneros *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Streptococcus* y *Leuconostoc* (Fuglsang et al., 2003; Muguerza et al., 2006; Ramchandran et al., 2008; Nielsen et al., 2009; Pihlanto et al., 2010). Sin embargo, existe otro grupo de microorganismos proteolíticos, las levaduras, cuya capacidad para producir hidrolizados y péptidos antihipertensivos a partir de proteínas lácteas no había sido prácticamente explorada.

Las levaduras son microorganismos eucariotas capaces de colonizar ambientes variados ricos en compuestos de carbono (Bourgeois et al., 1994). Aunque la levadura más estudiada es *S. cerevisiae*, existe otro grupo de levaduras que juegan un papel clave en los procesos de fabricación de productos lácteos: las levaduras no convencionales o levaduras no-*Saccharomyces* (Abbas, 2006; De Freitas et al., 2009). Se eligieron tres especies, *K. lactis*, *K. marxianus* y *D. hansenii*, porque además de ser consideradas levaduras GRAS (Generally Recognized As Safe), son típicas de productos lácteos. El género *Kluyveromyces*, y particularmente la especie *K. lactis*, es considerada como un modelo genético para estudios básicos, además de una levadura industrial fuente de distintos metabolitos y enzimas (Pariza y Johnson, 2001). La especie *K. marxianus*, menos estudiada que *K. lactis*, se ha aislado de una gran

variedad de hábitats, lo que implica una diversidad metabólica alta y la posibilidad de su utilización para diferentes aplicaciones biotecnológicas, incluyendo la producción de enzimas, compuestos aromáticos (Fonseca et al., 2008) y etanol (Charoensophara et al., 2015), así como la producción de bioingredientes (Fonseca et al., 2008; Padilla et al., 2015). Ambas especies son fuentes importantes de actividad β-galactosidasa, y tradicionalmente se han empleado para producir productos bajos en lactosa y para el tratamiento biológico de sueros de quesería. Otra especie altamente heterogénea, y por lo tanto versátil, es *D. hansenii*, un objetivo atractivo para la aplicación biotecnológica (Hatoum et al., 2013; Johnson, 2013). *D. hansenii* es una de las levaduras predominantes en alimentos fermentados, desempeñando un papel importante en la elaboración de todo tipo de quesos (Padilla et al., 2014), produciendo etanol, CO_2 y diferentes compuestos volátiles como ésteres, cetonas, aldehídos y compuestos azufrados que contribuyen al sabor y al aroma final de los mismos (Gardini et al., 2001; Martín et al., 2003).

Las cepas de levaduras utilizadas en esta tesis fueron aisladas de quesos tradicionales elaborados en la Comunidad Valenciana, y fueron seleccionadas para este trabajo por sus capacidad de hidrólisis de CN (Padilla et al., 2014). Se evaluaron varias cepas de las tres especies estudiadas, ya que muchas de las características metabólicas de las levaduras son específicas de cepa. En principio, todas las cepas fueron capaces de crecer en medio con glucosa y con CN o LF como única fuente de nitrógeno. Todos los hidrolizados, con la excepción del hidrolizado de LF producido por *K. lactis* Kl7, fueron capaces de inhibir *in vitro* la ECA en mayor o menor grado, lo que permitió la selección de 4 hidrolizados de CN (producidos por las cepas Dh4, Dh8, Kl3 y Km2) y 5 de LF (Dh4, Dh6, Kl6, Kl8 y Km2).

Cabe destacar que dos de las cepas seleccionadas, Dh4 y Km2, fueron capaces de producir los hidrolizados con mayor potencia inhibitoria de la ECA independientemente de la proteína láctea utilizada. Además, los hidrolizados producidos por estas dos cepas provocaron los mayores descensos de la presión arterial sistólica (PAS) en SHRs. Resulta interesante que el efecto antihipertensivo *in vivo* de los hidrolizados de CN producidos con estas dos cepas fue muy similar al observado en la leche fermentada en la que se encuentran los tripéptidos VPP e IPP (-21,8 ± 4,2 mm de Hg) (Yamamoto y Takano, 1999). En el caso de los hidrolizados de LF, el efecto antihipertensivo más potente se observó con el hidrolizado producido con la cepa Km2, siendo éste superior al observado con hidrolizados de LF producidos enzimáticamente en nuestros estudios previos, bien con pepsina (-15,4 ± 3,8 mm de Hg) (Ruiz-Giménez et al., 2012) o con proteinasa K (-19 ± 7 mm Hg) (Fernández-Musoles et al, 2013b).

Por tanto, un resultado relevante de esta tesis es que se ha demostrado la capacidad del sistema proteolítico de *K. lactis*, *K. marxianus* y *D. hansenii* para hidrolizar CN y LF y liberar secuencias con efecto antihipertensivo. Además pone de manifiesto que la LF puede ser considerada una fuente de péptidos antihipertensivos al menos tan buena como la CN. Hasta la fecha, la utilización de levaduras apenas se había considerado para la obtención de péptidos antihipertensivos. En trabajos pioneros se encontraron los péptidos VPP e IPP en una leche fermentada que contenía *Lactobacillus helveticus* y *S. cerevisiae* (Nakamura et al., 1995a; Nakamura et al., 1995b). Sin embargo, posteriormente se asumió que el responsable de producir estos péptidos era *Lb. helveticus*, concretamente a través de la proteinasa extracelular CP790 (Yamamoto et al., 1994a; Yamamoto et al., 1994b). La capacidad de *K. marxianus,* sola o en cocultivo*,* para generar péptidos inhibidores de la ECA estaba

demostrada (Belem et al., 1999; Didelot et al., 2006; Chaves-López et al., 2012), pero no el efecto antihipertensivo *in vivo* de estas secuencias o leches fermentadas. Por tanto, nuestros resultados son los primeros en los que se demuestra, en un modelo animal preclínico, el efecto antihipertensivo de hidrolizados de proteínas producidos con levaduras no convencionales: confirma el potencial de *K. marxianus* y describe por primera vez a *K. lactis* y *D. hansenii* como productoras de péptidos antihipertensivos.

2. Las levaduras no convencionales producen péptidos antihipertensivos derivados de proteína lácteas con un residuo de Pro en el extremo C-terminal

Como la CN es la fuente principal de péptidos antihipertensivos (Martínez-Maqueda et al., 2012), en este trabajo no abordamos la identificación de nuevos péptidos presentes en los hidrolizados de CN con mayor potencia inhibitoria, sino la producción por levaduras no convencionales de secuencias peptídicas concretas cuyo efecto antihipertensivo ya había sido demostrado en modelos animales preclínicos y/o en ensayos clínicos con pacientes. Este escrutinio incluyó los lactotripéptidos VPP e IPP, las secuencias RYLGY y AYFYPEL, identificadas a partir de un hidrolizado enzimático de CN, y los péptidos LHLPLP y HLPLP producidos mediante fermentación con *Enterococcus faecalis,* y en el caso de este último, también mediante hidrólisis enzimática.

Nuestros resultados demuestran que sólo *D. hansenii* fue capaz de producir algunas de las secuencias antihipertensivas, en concreto LHLPLP y HLPLP. Además, esta capacidad es dependiente de cepa, ya que sólo 2

de las 23 estudiadas, generaron estos péptidos. Por tanto, la estrategia evaluada en esta tesis se sumaría a las ya descritas de fermentación con *E. faecalis* y a la hidrólisis enzimática empleando corolasa PP (Miguel et al., 2006: Hernández-Ledesma et al., 2007; Quirós et al., 2007). La ventaja de utilizar *D. hansenii*, levadura GRAS, frente a *E. faecalis* es indudable, ya que aunque esta última es una bacteria comensal común en el tracto digestivo, está relacionada con el desarrollo de ciertas infecciones, por lo que su uso en la industria alimentaria es controvertido (Franz et al., 2011). Sin embargo, hay que tener en cuenta que *D. hansenii* no es capaz de fermentar la lactosa, por lo que su empleo directamente en leche requeriría la inoculación conjunta por ejemplo con BAL o incluso con *Kluyveromyces*. Es interesante destacar que estas especies coexisten durante el proceso de fabricación de queso, donde *D. hansenii* suele ser la especie mayoritaria durante la maduración. Los péptidos LHLPLP y HLPLP también aparecieron en secuencias más largas (LHLPLPLLQS, LHLPLPL y HLPLPL) identificadas en el hidrolizado producido por la cepa Dh14. Algunas de estas secuencias también se identificaron en queso azul Valdeón (Sánchez-Rivera et al., 2014), sugiriendo la posible participación de *D. hansenii.* Serían necesarios más estudios para confirmar la contribución de esta levadura a la producción de péptidos antihipertensivos en productos lácteos fermentados.

A diferencia de la CN, hasta la realización de esta tesis doctoral sólo cinco péptidos derivados de LF con secuencias RRWQWR, WQ (Ruiz-Giménez et al., 2010), RPYL, LIWKL y LNNSRAP (Ruiz-Giménez et al., 2012) habían mostrado potencia inhibitoria de la ECA y efecto antihipertensivo después de la administración oral a SHRs. Los dos primeros derivan de LfcinB, y los tres restantes se identificaron en hidrolizados enzimáticos de LF. Sin embargo, no se identificó ninguna de estas secuencias en el hidrolizado de LF producido con *K. marxianus* Km2,

indicando que la estrategia utilizada permitió la obtención de hidrolizados antihipertensivos con perfiles enzimáticos diferentes a los ya descritos, y por tanto la identificación de nuevas secuencias antihipertensivas.

Los estudios *in vitro* de inhibición enzimática han permitido establecer relaciones entre la secuencia peptídica y la potencia inhibitoria. Sin embargo, aunque se han realizado numerosos estudios al respecto, siguen sin ser totalmente concluyentes, ya que además de la secuencia aminoacídica, también es importante la longitud del péptido y su potencial electrostático (Li et al., 2004; De Leo et al., 2009). Como se ha comentado previamente, los péptidos inhibidores de la 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 (Tyr, Phe y Trp), junto con residuos de Pro, Lys o Arg (Murray y FitzGerald, 2007). Además, la presencia de estos aminoácidos en el extremo C-terminal también ha sido descrita para péptidos opioides, antioxidantes, insulinotrópicos e inhibidores de la dipeptidil peptidasa IV (Teschemacher et al., 1997; FitzGerald et al., 2004; Nongonierma y FitzGerald, 2014; Nongonierma y FitzGerald, 2015).

También se ha descrito que cuando una secuencia peptídica tiene un residuo de Pro en la última o antepenúltima posición del extremo C-terminal muestra más afinidad para unirse al sitio activo de la ECA (Rohrbach et al., 1981), lo que podría deberse a la estructura rígida de este aminoácido, que tiene una conformación favorable para la interacción con el sitio activo de la enzima (Cushman et al., 1977). Este es el caso de las secuencias antihipertensivas identificadas en este trabajo, y justificaría la potencia inhibitoria de los péptidos derivados de LF encontrados en el hidrolizado producido con *K. marxianus* Km2 (DPYKLRP, PYKLRP, YKLRP y GILRP), así como con los péptidos antihipertensivos LHLPLP y HLPLP

derivados de CN generados por dos cepas de *D. hansenii*. Estos dos últimos péptidos, además de tener en el extremo C-terminal un residuo de Pro, también lo tienen en la antepenúltima posición, lo que podría aumentar su potencia inhibitoria.

Cabe destacar que las secuencias producidas por *K. marxianus* Km2 comparten el tripéptido C-terminal LRP, señalado mediante ensayos *in silico* como la secuencia derivada de LF con mayor potencial para inhibir la ECA (Vermeirssen et al., 2004), lo cual confirman nuestros resultados. Esta secuencia también se identificó en proteínas del endospermo de maíz (Miyoshi et al., 1995), y en β- y κ-CN de origen humano (Komura et al., 1989; Komura et al., 1990).

Dado que se identificaron las secuencias DPYKLRP, PYKLRP y YKLRP que difieren en su extremo N-terminal, se incluyeron también las secuencias KLRP y LRP en el estudio para establecer relaciones secuencia-potencia inhibitoria independientes del residuo de Pro en el extremo C-terminal. En este sentido, se ha indicado que no existe relación entre el extremo N-terminal y la potencia inhibitoria (Wu et al., 2006), aunque en el caso de péptidos derivados de LfcinB, sí se ha demostrado que ciertas elongaciones a partir del extremo N-terminal provocan un aumento de la potencia inhibitoria de la ECA (Ruiz-Giménez et al., 2010). Nuestros resultados sugieren que las elongaciones a partir del extremo N-terminal decrecen la potencia inhibitoria *in vitro*, aunque no el efecto antihipertensivo. Es interesante remarcar que el tripéptido LRP es la secuencia derivada de LF más potente descrita hasta la fecha, con un valor de IC_{50} (0,35 ± 0,03 μM) muy similar al descrito en los ensayos *in silico* (0,27 μM). Además, también se puede concluir que elongaciones a partir del extremo C-terminal del tripéptido LRP provocan un descenso de su potencia inhibitoria ya que para el péptido LRPVAA se describió un valor de IC_{50} = 4,14 μM (Lee et al., 2006). Este resultado confirmaría la

importancia del residuo de Pro en el extremo C-terminal. Recientemente se ha identificado en un hidrolizado de CN con efecto antihipertensivo el péptido MKP, con secuencia y potencia inhibidora de la ECA (IC_{50} = 0,43 μM) similares a nuestro tripéptido LRP. Además, MKP marcado radioactivamente se detectó en suero de SHRs tras su administración oral (Yamada et al., 2015).

La posible relevancia funcional de la secuencia LRP también vendría avalada por los resultados obtenidos en los ensayos de digestión gastrointestinal, ya que fue el único péptido resistente a las proteasas gástricas y pancreáticas. Además, el tripéptido se identificó en la mayoría de los digeridos, lo que sugiere su contribución al efecto antihipertensivo de los péptidos parentales. De hecho, nuestros resultados han demostrado que el efecto antihipertensivo de los péptidos producidos por *K. marxianus* no se correlaciona con su potencia *in vitro*: a pesar de diferencias superiores a dos órdenes de magnitud en los valores de potencia inhibitoria, todos los péptidos provocaron efecto antihipertensivo en SHRs. La liberación del fragmento mínimo antihipertensivo LRP podría explicar este comportamiento, aunque no se descarta la posibilidad de que estos péptidos actúen a través de diferentes mecanismos de acción. Independientemente de esta posibilidad, son necesarios más estudios que profundicen en la biodisponibilidad de los péptidos derivados de LF. En este trabajo sólo se ha abordado una digestión simulada *in vitro* cuyos productos de degradación pueden ser muy diferentes a los que se generarían *in vivo*. Las condiciones de la digestión *in vivo* díficilmente pueden ser reproducidas, debido a que además de las enzimas también intervienen otros factores como el sustrato, el tiempo de reacción, el pH, el transporte, etc. (Ekmekcioglu, 2002). Recientemente se ha publicado un protocolo de consenso para la digestión *in vitro* basado en condiciones fisiológicas con el objetivo de poder comparar resultados entre diferentes estudios (Minekus et al., 2014).

Se han descrito modificaciones del tripéptido LRP para mejorar su estabilidad. En primer lugar, se comprobó que el péptido sintético D-phg-LRP provocaba una reducción de la PAS de SHRs adelantada en el tiempo respecto a la causada por el tripéptido sin modificar. Por otro lado, ciclando este mismo tripéptido mediante un enlace disulfuro entre el aminoácido N-terminal y el C-terminal, se consiguió prolongar el efecto antihipertensivo del tripéptido sin modificar (Chen et al., 2003). Estos resultados *in vivo* muestran la posibilidad de usar estas técnicas para conseguir una mayor biodisponibilidad de los péptidos antihipertensivos.

En esta tesis hemos puesto de manifiesto el potencial de las proteasas de *D. hansenii* y *K. marxianus* para generar péptidos antihipertensivos que comparten un residuo de Pro en el extremo C-terminal. Además, en los hidrolizados de CN producidos con *D. hansenii* más del 60% de las secuencias identificadas tienen este residuo en el extremo C-terminal. Esta abundancia de péptidos con un residuo de Pro en el extremo C-terminal podría indicarnos similitudes entre las proteasas extracelulares de *K. marxianus* y *D. hansenii*. En general, hay poca información sobre las proteasas extracelulares de levadura responsables de la degradación inicial de CN y LF. En el caso de las BAL se sabe que poseen proteasas ligadas a la pared celular que pueden hidrolizar parcialmente la CN en péptidos asimilables que son degradados posteriormente por peptidasas de la membrana y del citoplasma (García-Garibay et al., 1993). En nuestro trabajo detectamos por zimografía actividad proteolítica en los sobrenadantes de los hidrolizados de CN producidos por las cepas de *D. hansenii* Dh1 y Dh14. En ambos sobrenadantes aparecía una banda de aproximadamente 50 kDa de masa molecular que podría ser la responsable de la degradación inicial de la CN. Además, en el sobrenadante de la cepa Dh1 apareció una segunda banda de peso molecular ligeramente más alto, lo que parece sugerir un perfil

proteolítico diferente para ambas cepas. Hasta el momento se han identificado pocas proteasas extracelulares provenientes de levaduras no-convencionales. Se purificó una proteasa extracelular de masa molecular aproximada de 45 kDa en los cultivos de *K. marxianus* IFO 0288 y que fue caracterizada como una serín proteasa (Foukis et al., 2012). Las propiedades que posee esta enzima sugieren que *K. marxianus* representa una fuente importante de proteasas extracelulares que, por su condición de levadura GRAS, pueden tener un gran interés biotecnológico. En nuestro caso no se abordó la caracterización del perfil extracelular proteolítico de *K. marxianus*, aunque trabajos posteriores del grupo apuntan a la existencia de al menos dos proteasas: una que coincidiría con la banda de 50 kDa observada en los sobrenadantes de *D. hansenii*, y otra banda de menor tamaño que no se había observado previamente. En estos momentos se dispone del genoma secuenciado de ambas especies (http://genolevures.org), lo que sin lugar a dudas facilitará la identificación y posterior caracterización de las proteasas implicadas en la generación de péptidos antihipertensivos derivados de proteínas lácteas.

3. La inhibición de la enzima conversora de angiotensina (ECA) es uno de los principales mecanismos de acción de los péptidos antihipertensivos derivados de lactoferrina (LF)

Aunque los péptidos derivados de LF identificados en el hidrolizado producido por *K. marxianus* mostraron un efecto antihipertensivo en SHRs bastante similar, destacaron el heptapéptido DPYKLRP y el tripéptido LRP por provocar unos efectos de bajada de la PA similares al causado por el captopril, y por su capacidad de mantener este efecto durante 24 h. Además, se observó una dosis-dependencia durante todo el efecto

antihipertensivo. Estos resultados nos llevaron a profundizar en el mecanismo de acción responsable de su efecto .

A pesar de los numerosos resultados que demuestran la potencia inhibitoria *in vitro* sobre la ECA de los péptidos derivados de proteínas alimentarias, la demostración de la inhibición *in vivo* de la enzima no es habitual. Por ello, decidimos determinar diferentes componentes del SRAA en el suero de las SHRs tras la administración oral de DPYKLRP y LRP. Nuestros resultados demuestran que la inhibición de la ECA sería el principal mecanismo subyacente a su efecto antihipertensivo, basado en la reducción no solo de los niveles de ECA circulantes sino también en los de Ang II, su producto de reacción. Es interesante destacar que la reducción de la actividad de la ECA y de los niveles de Ang II causada por la administración del heptapéptido fueron superiores a los provocados por LRP, y similares a los producidos por el captopril. Estos resultados concuerdan con los que obtuvo nuestro grupo de trabajo tras administrar de forma crónica a SHRs un hidrolizado de LF obtenido con pepsina, observando reducciones en la actividad de la ECA, de los niveles de Ang II y de aldosterona, así como un aumento compensatorio de la actividad de la renina (Fernández-Musoles et al., 2013a). En esta tesis no se observaron cambios significativos en los niveles de aldosterona, hormona esteroidea cuya secreción adrenal endocrina aumenta en presencia de Ang II (Carey y Siragy, 2003). Esto se debe probablemente a la administración de una dosis unica, ya que tampoco se observó en el caso del captopril. Recientemente se ha descrito el efecto antihipertensivo de las secuencias IQW y LKP, derivadas de huevo, acompañado de una reducción en los niveles circulantes de Ang II tras administración crónica a SHRs (Majumder et al., 2015). Por tanto, estas secuencias regularían *in vivo* el SRAA a través de la inhibición de la ECA, al igual que nuestros péptidos DPYKLRP y LRP.

Otra estrategia para investigar el mecanismo de acción *in vivo* de los péptidos antihipertensivos fue inducir la hipertensión en ratas normotensas Wistar-Kyoto (WKY), infundiendo de forma continua Ang I o Ang II. Esta aproximación permite diferenciar los efectos de los péptidos sobre la hipertensión inducida con Ang I y por tanto dependiente de la ECA, o sobre la hipertensión inducida con Ang II independiente de la ECA y mediada directamente por la activación de los receptores AT_1. Esta metodología, cuya aplicación no es frecuente en el estudio de péptidos de origen alimentario, nos ha permitido confirmar la inhibición *in vivo* de la ECA causada por el heptapéptido DPYKLRP y, en menor medida, por el tetrapéptido antihipertensivo RPYL derivado de LF (Ruiz-Giménez et al., 2012), ya que ambos revirtieron la hipertensión inducida por Ang I. En este estudio no se incluyó el tripeptido LRP porque sus efectos siempre habían sido similares a los del heptapéptido, por lo que podíamos suponer también un efecto parecido en estos ensayos. Además, tuvimos en cuenta que cuantitativamente los efectos de DPYKLRP sobre la inhibición de la ECA fueron ligeramente superiores a los de LRP. En su lugar se evaluó la secuencia RPYL, cuyo efecto antihipertensivo, aunque menor en magnitud y duración que el provocado por el heptapéptido, ya se había demostrado en SHRs (Ruiz-Giménez et al., 2012). En concordancia con estos resultados, la reversión causada por RPYL fue menor que la provocada por DPYKLRP en los ensayos de hipertensión inducida con Ang I.

Otra de las razones que nos llevó a incluir a RPYL en este estudio fueron los datos previos que sugerían su capacidad de bloqueo de los receptores AT_1 en ensayos *ex vivo* con arterias aisladas y también en ensayos *in vitro* de unión ligando-receptor (Fernandez-Musoles et al., 2014). Sin embargo, estos resultados no pudieron confirmarse *in vivo* con los ensayos de hipertensión inducida por Ang II, lo que vuelve a poner de manifiesto la falta de correlación entre los ensayos *in vitro* e *in vivo* y señala otra vez el papel clave que jugaría la biodisponibilidad. Sí se

observó, por el contrario, un ligero efecto de DPYKLRP sobre la hipertensión inducida por Ang II, sugiriendo que esta secuencia tiene dos dianas en el SRAA: la ECA y los receptores AT_1.

En su conjunto, estos resultados ponen de manifiesto la inhibición de la ECA como principal mecanismo de acción de los péptidos derivados de LF, y apuntan a un papel menos relevante para el bloqueo de los receptores de Ang II.

Por último, la actividad renina, otro de los componentes principales del SRAA, no se ha estudiado en esta tesis. En los ensayos *in vivo* con DPYKLRP y LRP no se hicieron determinaciones de actividad renina, por lo que no se pudo confirmar el aumento compensatorio debido a la inhibición de la ECA que se observó tras la administración crónica de un hidrolizado de LF (Fernández-Musoles et al., 2013a), y que es característico de los inhibidores de la enzima (Brunner y cols., 1993). Es interesante destacar que en los últimos años se han descrito un buen número de péptidos e hidrolizados de distinto origen capaces de inhibir *in vitro* la actividad renina (Aluko, 2015), aunque sólo con un hidrolizado de semillas de cáñamo se obtuvo confirmación *in vivo* tras su administración crónica a SHRs, observándose una reducción de los niveles de actividad renina y de la ECA plasmáticas (Girgih et al., 2014a). Hasta el momento no se ha podido establecer ninguna correlación entre las actividades inhibidoras de la renina y de la ECA de hidrolizados y péptidos, lo que sugiere que el modo de acción de ambos tipos de péptidos inhibidores sería diferente (Aluko, 2015).

4. Los péptidos derivados de lactoferrina (LF) ejercen su efecto a través de diferentes sistemas de regulación de la presión arterial (PA)

Son numerosos los ejemplos tanto de péptidos naturales como de fármacos que, siendo inhibidores de la ECA, también tienen efecto sobre otras dianas de sistemas reguladores de la PA diferentes al SRAA, demostrando que no siempre su efecto antihipertensivo está relacionado solamente con esta capacidad inhibitoria. En el caso concreto de los péptidos derivados de LF, nuestro grupo ya había señalado la posible relevancia del sistema endotelina (Fernández-Musoles et al., 2013b).

Por todo ello, en esta tesis doctoral se profundizó en el mecanismo de acción de los péptidos derivados de LF. Dadas las evidencias que señalan las múltiples dianas de los péptidos antihipertensivos, decidimos, abordar una aproximación global que revelara la acción de los péptidos a través de los distintos sistemas reguladores de la PA, en lugar de centrarnos en dianas individuales. La estrategia elegida fue una aproximación transcriptómica que permitió estudiar el efecto de los péptidos derivados de LF sobre la expresión génica de un conjunto de 84 genes relacionados con el SRAA, sistema endotelina (ET), señalización y metabolismo de óxido nítrico (NO), síntesis y señalización de cGMP, señalización mediada por segundos mensajeros, transporte de iones y metabolismo lipídico. Como modelo biológico se eligieron células endoteliales, que tapizan la superficie interna de los vasos sanguíneos y se consideran un buen modelo para estudiar los mecanismos moleculares implicados en la regulación de la PA. En concreto se trabajó con células HUVEC, que tratamos durante 24 h con un hidrolizado de LF obtenido con pepsina.

Nuestros resultados señalan que los péptidos derivados de LF no afectaron la expresión de los genes que codifican distintos componentes

del SRAA, pero sí se observó inhibición de la actividad de la ECA en los sobrenadantes de las células tratadas con el hidrolizado de LF. Estos resultados son similares a los descritos tras la administración oral de IPP y VPP a SHRs, ya que no se observaron cambios significativos en la expresión de genes implicados en el SRAA (Yamaguchi et al., 2009). En nuestro trabajo, tampoco se vió afectada la expresión de los genes del sistema ET, aunque una de las dianas del hidrolizado de LF es la actividad ECE (Fernández-Musoles et al., 2013b). A pesar de que los modelos utilizados son diferentes, los resultados parecen sugerir que los péptidos derivados de LF actuarían como inhibidores de las actividades ECA o ECE, pero no afectarían a la expresión de los genes que codifican las enzimas. De todas formas, para descartar estos efectos sería necesario evaluar la dependencia de estos efectos respecto de la concentración del hidrolizado y también observar a tiempos más cortos donde sí pudiera haber un efecto sobre la expresión génica.

En esta tesis se ha demostrado que el hidrolizado de LF modifica la expresión de genes relacionados con el metabolismo y señalización de NO. En concreto, se reprime la expresión de NOSTRIN, una proteína que modula la distribución subcelular de la óxido nítrico sintasa endotelial (eNOS) y por lo tanto la liberación de NO. La sobreexpresión de NOSTRIN promueve la traslocación de la eNOS desde la membrana plasmática a vesículas intracelulares, lo que se traduce en una reducción de la actividad enzimática, y por tanto de NO (Zimmermann et al., 2002). Por el contrario, una reducción de la expresión de NOSTRIN, como la causada por el hidrolizado de LF, contribuiría a un incremento de la producción de NO en células endoteliales (McCormick et al., 2011). De acuerdo con la represión de NOSTRIN, nuestros resultados muestran también una reducción en los niveles proteicos de NOSTRIN, y un incremento de la producción de NO. Por tanto, NOSTRIN sería una diana molecular de los péptidos derivados

de LF, que como consecuencia provocan un aumento en la concentración de NO en células endoteliales. Se han descrito efectos vasorelajantes de péptidos antihipertensivos derivados de proteínas alimentarias. Es el caso de péptidos derivados de huevo, leche y soja, que estimulan la síntesis de NO en arterias aisladas de rata (Matoba et al., 1999; Sipola et al., 2002; García-Redondo et al., 2010; Hirota et al., 2011) y en células endoteliales humanas (Ringseis et al., 2005; Hirota et al., 2011). En algunos estudios se ha señalado la fosforilación de la eNOS (Shimizu et al., 2010; Ko et al., 2012) o un incremento de su expresión (Sánchez et al., 2011) como responsables de la mayor síntesis de NO. En cambio, es la primera vez que NOSTRIN se señala como responsable, al menos en parte, de los efectos antihipertensivos causados por péptidos. Además, el aumento de NO en las células HUVEC se vió acompañado con un aumento en la expresión génica de GUCY1, que codifica la guanilato ciclasa soluble (GCs), el receptor fisiológico del NO y responsable de la síntesis de cGMP, molécula que desencadena una cascada de señalización intracelular que conduce a la vasorelajación (Schmidt et al., 1993).

Otro resultado interesante del estudio de expresión génica es la posible contribución de las prostaglandinas al efecto antihipertensivo de los péptidos derivados de LF. Hemos observado un incremento en la expresión del gen COX-2 que codifica una de las enzimas implicadas, junto con COX-1, en la conversión del ácido araquidónico a prostaglandinas, tromboxano A2 y prostaciclina. Aunque el principal papel de COX-2 está relacionado con la vasoconstricción debido a la síntesis de tromboxano, se ha descrito que en humanos sanos genera principalmente prostaciclina vasodilatadora (McAdam et al., 1999). Además, el efecto observado viene avalado por el hecho de que la expresión de COX-2 y los niveles de prostaciclina también se incrementan durante tratamientos con fármacos inhibidores de la ECA (Kohlstedt et al., 2005).

Finalmente, resaltar que los estudios de expresión génica como el abordado en esta tesis no son frecuentes en el caso de péptidos antihipertensivos derivados de proteínas alimentarias. Cuando se inició este trabajo, se había descrito el efecto de la administración crónica de VPP e IPP a SHRs sobre la expresión génica en aorta utilizando micromatrices de DNA (Yamaguchi et al., 2009). Los resultados demostraron cambios tenues en la expresión de genes asociados con la función vascular, destacando la sobreexpresión del gen que codifica la eNOS, lo que ha sido confirmado en un estudio reciente donde en lugar de administrar los lactotripéptidos se utilizó una leche fermentada con *Lb. helveticus* (Chen et al., 2015).

Parece claro que este tipo de estudios mediante aproximaciones globales, aunque todavía escasos, señalan distintas dianas moleculares en las rutas del NO y prostaglandinas. Además, muestran la complejidad de los efectos causados por los péptidos antihipertensivos, cuya caracterización inicial los definió como inhibidores de la ECA. Es de esperar que la futura aplicación de las metodologías ómicas proporcionará mayor información sobre el mecanismo de acción de los péptidos antihipertensivos, y por tanto abrirá nuevas vías en el tratamiento preventivo o paliativo de la hipertensión. Desde el punto de vista de la alimentación funcional, la definición del mecanismo de acción de los péptidos antihipertensivos facilitará, sin lugar a dudas, la obtención de declaración saludable (De Noni et al., 2009; Agostoni et al., 2012).

5. Consideraciones finales

Esta tesis ha demostrado el potencial de los péptidos derivados de LF en el control de la hipertensión. Estos péptidos pueden liberarse de la proteína parental no sólo mediante hidrólisis enzimática sino también utilizando levaduras proteolíticas clasificadas como seguras. Además, algunas de estas levaduras representan una estrategia alternativa para generar péptidos antihipertensivos derivados de CN.

Las diferentes aproximaciones experimentales utilizadas en esta tesis, principalmente ensayos *in vitro* e *in vivo*, señalan al SRAA como diana de estos péptidos, principalmente la ECA, y de forma secundaria los receptores AT_1. La inhibición de la renina, otra enzima clave del sistema no se ha abordado en esta tesis, aunque cada vez aparecen más estudios que ponen en evidencia su importancia. A pesar de la caracterización previa de ciertos péptidos derivados de LF como inhibidores de la ECE, en el estudio transcriptómico abordado en este trabajo no se ha puesto de manifiesto la implicación del sistema ET; sin lugar a dudas debe insistirse en la exploración de estas posibles dianas. Por último, el modelo *in vitro* HUVEC ha demostrado que un hidrolizado de LF es capaz de incrementar la producción de NO y modificar la expresión de genes relacionados con la hipertensión. En concreto, la proteína NOSTRIN sería la diana molecular de estos péptidos.

En conjunto, se han puesto de manifiesto diferentes mecanismos a través de los cuales los péptidos derivados de LF podrían ejercer su efecto antihipertensivo (Manzanares et al., 2015), lo que incrementa el potencial de la LF como fuente de péptidos multifuncionales con efecto antihipertensivo. Este hecho representa un valor añadido para la LF, cuyas aplicaciones nutraceúticas se basan principalmente en sus propiedades anticancerígenas, antiinflamatorias, moduladoras de la inmunidad y

antimicrobianas (García-Montoya et al., 2012).

Desde el punto de vista de la aplicación de los péptidos derivados de LF como agentes antihipertensivos orales, los ensayos clínicos serán, sin lugar a dudas, la próxima meta a alcanzar. Finalmente hay que recordar la importancia de los estudios de biodisponibilidad, la identificación del fragmento activo capaz de alcanzar la diana molecular en el organismo, así como evaluar las características de absorción, distribución, metabolismo y excreción. En este contexto, resulta imprescindible la aplicación de técnicas analíticas avanzadas que sorteen las limitaciones asociadas a la detección de péptidos de pequeño tamaño en los complejos fluidos biológicos (Le Maux et al., 2015).

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Conclusiones

1. Los péptidos derivados de la proteína láctea lactoferrina (LF), obtenidos bien por hidrólisis enzimática o mediante el uso de levaduras, confirman a nivel preclínico su potencial para controlar la hipertensión, mediante mecanismos que no se limitan a la inhibición de la enzima conversora de angiotensina (ECA).

2. Levaduras GRAS de las especies *Debaryomyces hansenii*, *Kluyveromyces lactis* y *Kluyveromyces marxianus* pueden utilizarse para producir hidrolizados de LF y de caseína (CN) con efecto antihipertensivo en ratas espontáneamente hipertensas (SHRs).

3. Las cepas *D. hansenii* Dh1 y Dh14 producen los péptidos antihipertensivos LHLPLP y HLPLP, que pueden ser en parte responsables de la inhibición de la ECA de los hidrolizados de CN producidos con *D. hansenii*.

4. La cepa *K. marxianus* Km2 produce nuevos péptidos derivados de LF que comparten el tripéptido LRP en su extremo C-terminal. Estas secuencias provocan la inhibición *in vitro* de la ECA y efectos antihipertensivos en SHRs tras su administración oral.

5. El efecto antihipertensivo en SHRs provocado por las secuencias DPYKLRP, PYKLRP, YKLRP, KLRP y GILRP podría deberse a la generación del tripéptido LRP, que sería el fragmento mínimo responsable del efecto antihipertensivo de los péptidos parentales.

6. La inhibición de la ECA, es el principal mecanismo de acción responsable de los efectos antihipertensivos de los péptidos DPYKLRP, LRP y RPYL. El bloqueo de los receptores AT_1 también contribuye, aunque con menor relevancia, al efecto antihipertensivo del heptapéptido DPYKLRP.

7. El hidrolizado enzimático de LF producido con pepsina fue capaz de inhibir la ECA, modificar la expresión de genes relacionados con la hipertensión y aumentar la producción de NO en células endoteliales humanas. La represión de NOSTRIN, tanto a nivel de mRNA como de proteína, podría ser responsable del aumento de la producción de NO en el endotelio.

8. El mecanismo de acción de los péptidos antihipertensivos derivados de LF es complejo, implicando a distintos sistemas de regulación de la presión arterial (PA). Además de la inhibición de la ECA, la interacción con otros componentes del sistema renina angiotensina aldosterona (SRAA) y del sistema del óxido nítrico (NO) serían responsables del efecto antihipertensivo de dichos péptidos.

Difusión de resultados

Food Research International 53 (2013) 203–208

Contents lists available at SciVerse ScienceDirect

Food Research International

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Dairy yeasts produce milk protein-derived antihypertensive hydrolysates

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ARTICLE INFO

Article history:
Received 1 March 2013
Accepted 1 May 2013
Available online 9 May 2013

Keywords:
Debaryomyces hansenii
Kluyveromyces lactis
Kluyveromyces marxianus
Casein-derived peptides
Lactoferrin-derived peptides
ACE inhibition

ABSTRACT

The potential of 20 dairy yeast strains belonging to *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Kluyveromyces marxianus* species was examined for the production of milk protein-derived antihypertensive hydrolysates. For this purpose yeast strains were grown in microbiological medium with casein or lactoferrin as sole nitrogen source, and the inhibitory effects of casein and lactoferrin hydrolysates (CSHs and LFHs) on angiotensin I-converting enzyme (ACE) activity were determined. Based on the ACE-inhibitory activity, four CSHs and five LFHs were selected, and permeate fractions with molecular masses lower than 3 kDa (pCSHs and pLFHs) were obtained. *In vitro* ACE-inhibitory potencies (IC_{50}) of permeates varied from 18.8 to 87.6 μg/ml (pCSHs) and from 50.2 to 500 μg/ml (pLFHs). *K. marxianus* Km2 strain grown on either casein or lactoferrin produced the most potent permeates. pCSHs and pLFHs were orally administered to spontaneously hypertensive rats (SHRs) and exerted *in vivo* antihypertensive effect. In conclusion, the present study contributes to a better insight into bioactive compounds produced by dairy yeasts and shows the feasibility of selected yeasts to produce orally effective antihypertensive milk protein-derived hydrolysates.

1. Introduction

Food derived bioactive peptides are attracting increasing interest because of their variety and multifunctionality. Undoubtedly, those with blood pressure-lowering effects are receiving increasing attention due to the worldwide growing prevalence of hypertension (Kearney et al., 2005). One of the main targets for the treatment of hypertension is the renin angiotensin system (RAS), and its inhibition at three possible levels, angiotensin-converting enzyme (ACE), upstream renin activity or downstream angiotensin receptors, is the pharmacological basis for commonly used antihypertensive drugs (Fragasso et al., 2012). ACE, which hydrolyzes both the inactive angiotensin I into vasoconstrictor angiotensin II and the vasodilator bradykinin into an inactive metabolite, is also the main target for antihypertensive food-derived peptides developed as an alternative to drugs (reviewed in Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012).

Nowadays milk proteins are the main source of antihypertensive peptides. Among the approaches for releasing ACE-inhibitory peptides from intact milk proteins, fermentation with proteolytic lactic acid bacteria (LAB) to partially digest the caseins during the manufacture of dairy products is a successful strategy (reviewed in Hernández-Ledesma, Contreras, & Recio, 2011). Moreover, milk fermented with *Lactobacillus helveticus*, containing the casein-derived peptides VPP and IPP, has shown significant antihypertensive effects in rats and humans (Hata et al., 1996; Nakamura, Yamamoto, Sakai, & Takano, 1995; Seppo, Jauhiainen, Poussa, & Korpela, 2003; Seppo, Kerojoki, Suomalainen, & Korpela, 2002). Also enterococci from dairy origin are able to hydrolyse casein into peptides with ACE-inhibitory activity and antihypertensive effect (Chaves-López et al., 2011; Muguerza et al., 2006); however the pathogenic potential of some *Enterococcus* strains (Franz, Holzapfel, & Stiles, 1999) may hamper their use in food production.

Yeast products have been used for many years as ingredients and additives in food processing, although their potential bioactivity has been less investigated (Abbas, 2006; Dawson, 2002). Yeasts isolated from dairy environments have proteolytic character (Jakobsen & Narvhus, 1996) and thus potential for releasing bioactive peptides. *Kluyveromyces marxianus* was pointed out as a promise candidate to generate antihypertensive peptides from the whey proteins α-lactalbumin and β-lactoglobulin, alone (Belem, Gibbs, & Lee, 1999) or in combination with *Lactobacillus rhamnosus* (Hamme, Sannier, Piot, & Bordenave-Juchereau, 2009; Hamme, Sannier, Piot, Didelot, & Bordenave-Juchereau, 2009). Recently *K. marxianus* isolated from

Abbreviations: ACE, angiotensin I-converting enzyme; CSH, casein hydrolysate; GRAS, generally recognized as safe; LAB, lactic acid bacteria; LFH, lactoferrin hydrolysate; pCSH, casein hydrolysate permeate with molecular mass lower than 3 kDa; pLFH, lactoferrin hydrolysate permeate with molecular mass lower than 3 kDa; RAS, renin angiotensin system; RP-HPLC, reversed phase-high performance liquid chromatography; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat; TFA, trifluoroacetic acid.

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0963-9969/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodres.2013.05.005

LWT - Food Science and Technology 61 (2015) 550–556

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Dairy *Debaryomyces hansenii* strains produce the antihypertensive casein-derived peptides LHLPLP and HLPLP

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ARTICLE INFO

Article history:
Received 21 May 2014
Received in revised form
11 November 2014
Accepted 4 December 2014
Available online 11 December 2014

Keywords:
Dairy yeasts
Debaryomyces hansenii
Kluyveromyces lactis
Kluyveromyces marxianus
Casein-derived antihypertensive peptides

ABSTRACT

The ability of dairy *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Kluyveromyces marxianus* strains to release the casein-derived antihypertensive sequences RYLGY, AYFYPEL, LHLPLP, HLPLP, VPP and/or IPP was examined. Yeast strains were grown in medium with casein as sole nitrogen source and the yeast casein hydrolysates (CSHs) were analysed by HPLC-MS/MS to search for the six antihypertensive sequences. Only LHLPLP and HLPLP were identified in CSHs and exclusively in *D. hansenii* Dh1 and Dh14 hydrolysates in which both antihypertensive sequences represented approximately 6 (CSH Dh1) and 10% (CSH Dh14) of total peptide content. In addition, a complete analysis of selected CSHs by HPLC-MS/MS allowed the identification of 35 (Dh1) and 46 (Dh14) additional peptides, which could also contribute to the observed *in vitro* ACE inhibitory potency of both hydrolysates (Dh1, $IC_{50} = 13.6 \pm 1.8$ μg/mL; Dh14, $IC_{50} = 17.5 \pm 2.1$ μg/mL) and might confer them multifunctional properties. Finally casein zymography revealed the presence of at least one extracellular protease with a molecular mass of about 50 kDa. In conclusion, the present study contributes to a better insight into bioactive compounds produced by dairy yeasts and shows the feasibility of *D. hansenii* strains to produce antihypertensive casein-derived peptides.

1. Introduction

Casein is an excellent substrate to produce peptides with angiotensin I converting enzyme (ACE)-inhibitory and antihypertensive effects (Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012; Otte, Shalaby, Zakora, Pripp, & El-Shabrawy, 2007). Basically, processing of milk proteins with food grade proteolytic preparations or fermentation of milk with lactic acid bacteria (LAB) proteolytic starters have been employed to release antihypertensive peptides. Both approaches have conducted to the development of commercial products based on milk proteins with antihypertensive effects in humans. In fact, one of the most popular functional foods contain the casein-derived ACE-inhibitory tripeptides VPP and IPP, which can be obtained by means of either milk fermentation (Nakamura, Yamamoto, Sakai, & Takano, 1995) or enzymatic hydrolysis using microbial proteases (Mizuno, Nishimura, Matsuura, Gotou, & Yamamoto, 2004).

We have previously identified casein-derived sequences with antihypertensive activity using both methods. Two novel peptides of sequences RYLGY and AYFYPEL were identified from a peptic casein hydrolysate (Contreras, Carrón, Montero, Ramos, & Recio, 2009). Both exerted *in vitro* inhibitory effects on ACE activity (IC_{50} values of 0.71 and 6.58 μM, respectively) and effectively decreased systolic blood pressure after oral administration to spontaneously hypertensive rats (SHRs) at 5 mg/kg of body weight. With respect to the fermentation strategy, the antihypertensive sequence LHLPLP was

Abbreviations: ACE, angiotensin I-converting enzyme; CSH, casein hydrolysate; GRAS, generally recognized as safe; LAB, lactic acid bacteria; RP-HPLC-MS/MS, reversed phase-high performance liquid chromatography tandem mass spectrometry; RP-HPLC-UV-MS, reversed-phase high performance liquid chromatography-UV and mass spectrometry; SHR, spontaneously hypertensive rat.

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http://dx.doi.org/10.1016/j.lwt.2014.12.019
0023-6438/

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Novel Antihypertensive Lactoferrin-Derived Peptides Produced by *Kluyveromyces marxianus*: Gastrointestinal Stability Profile and *In Vivo* Angiotensin I-Converting Enzyme (ACE) Inhibition

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ABSTRACT: Novel antihypertensive peptides released by *Kluyveromyces marxianus* from bovine lactoferrin (LF) have been identified. *K. marxianus* LF permeate was fractionated by semipreparative high performance liquid chromatography and 35 peptides contained in the angiotensin I-converting enzyme (ACE)-inhibitory fractions were identified by using an ion trap mass spectrometer. On the basis of peptide abundance and common structural features, six peptides were chemically synthesized. Four of them (DPYKLRP, PYKLRP, YKLRP, and GILRP) exerted *in vitro* inhibitory effects on ACE activity and effectively decreased systolic blood pressure after oral administration to spontaneously hypertensive rats (SHRs). Stability against gastrointestinal enzymes suggested that the sequence LRP could contribute to the *in vivo* effects of parental peptides. Finally, there were reductions in circulating ACE activity and angiotensin II level in SHRs after either DPYKLRP or LRP intake, thus confirming ACE inhibition as the *in vivo* mechanism for their antihypertensive effect.

KEYWORDS: *Kluyveromyces marxianus, lactoferrin-derived peptides, gastrointestinal digestion, antihypertensive effect, in vivo ACE inhibition*

INTRODUCTION

In the past decade, much work has been done to characterize the antihypertensive effects of peptides derived from food proteins.[1] Angiotensin I-converting enzyme (ACE) inhibition is the main target for those peptides. ACE, as part of the renin-angiotensin system (RAS), hydrolyzes both the inactive angiotensin I into vasoconstrictor angiotensin II and the vasodilator bradykinin into an inactive peptide leading to blood pressure upregulation.[2] The *in vitro* inhibitory effect of food protein-derived peptides on ACE activity is well established in contrast with the limited *in vivo* evidence available for the mechanism of action underlying their blood pressure lowering effect. Bioavailability of ACE-inhibitory peptides has also been studied intensively since it is known that bioactive peptides may undergo physiological transformations that determine their activity in the organism.[3] Most research has been focused on milk-derived antihypertensive peptides, some of which have shown beneficial effects in clinical assays, as reported in different meta-analyses.[4]

The use of the proteolytic system of lactic acid bacteria (LAB) to hydrolyze milk proteins is a successful strategy to release antihypertensive peptides.[5] By contrast few studies exploit the proteolytic potential of yeasts despite their contribution to proteolysis in dairy products is well established. In this context, the lactose-fermenting yeast *Kluyveromyces marxianus* regularly found in milk and dairy products has been pointed out as a promising candidate to generate antihypertensive peptides from the whey proteins α-lactalbumin and β-lactoglobulin.[6] Its potential to produce fermented milk with casein-derived ACE-inhibitory peptides has been also described,[7] although *in vivo* antihypertensive effects were not evaluated in any of these reports.

Bovine lactoferrin (LF), a well-characterized component of milk whey, is also a good source of antihypertensive peptides. We have shown that enzymatic LF hydrolyzates lower blood pressure and thus exhibit potential as orally effective antihypertensive compounds.[8,9] Moreover, after long-term intake of a pepsin LF hydrolyzate, there were reductions of circulating ACE activity, angiotensin II and aldosterone levels, as well as a compensatory increase of renin activity.[10] So far, only five LF-derived peptides with sequences RRWQWR, WQ,[11] RPYL, LIWKL and LNNSRAP[8] have shown antihypertensive effects after oral administration to spontaneously hypertensive rats (SHRs), although based on *in silico* studies

Received: November 29, 2013
Revised: January 29, 2014
Accepted: January 30, 2014
Published: January 30, 2014

Journal of Functional Foods 15 (2015) 294–300

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/jff

In vivo antihypertensive mechanism of lactoferrin-derived peptides: Reversion of angiotensin I- and angiotensin II-induced hypertension in Wistar rats

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ARTICLE INFO

Article history:
Received 19 December 2014
Received in revised form 14 March 2015
Accepted 18 March 2015
Available online

Keywords:
Antihypertensive peptides
Lactoferrin-derived peptides
Angiotensin-induced hypertension
Wistar rat
In vivo ACE inhibition
Renin angiotensin system

ABSTRACT

Novel peptides with antihypertensive effects in SHR rats have previously been identified in lactoferrin (LF) hydrolysates. To investigate their *in vivo* antihypertensive mechanism, we have assessed the blood pressure lowering effects of two of these LF-derived peptides (RPYL and DPYKLRP) in Wistar rats subjected to either angiotensin I- or angiotensin II-induced hypertension. Blood pressure was measured by the tail-cuff method, hypertension was induced by subcutaneous infusion of angiotensins, and then captopril, valsartan or LF-derived peptides orally administered. Angiotensin I- and angiotensin II-induced hypertension were reversed by captopril and valsartan, respectively. RPYL and DPYKLRP reversed angiotensin I-induced hypertension, while DPYKLRP but not RPYL produced a modest reversion of angiotensin II-elicited hypertension. Neither RPYL nor DPYKLRP modified normotension. Thus, *in vivo* ACE inhibition is involved in the antihypertensive effects of LF-derived peptides like RPYL and DPYKLRP, while inhibition of AT_1 receptor-mediated vasoconstriction plays a less relevant role.

1. Introduction

Hypertension is an important modifiable risk factor for cardiovascular disease, and its management includes not only pharmacological treatment but also lifestyle changes like physical activity and dietary habits (Ruilope, 2011). The increasing perception about the relationship between food and health is fostering the development of functional foods providing health benefits beyond nutrition (Roberfroid, 2002). Some dietary proteins contain embedded peptides that once released behave as bioactive peptides with different health-promoting properties including blood pressure lowering effects (Hartmann & Meisel, 2007).

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Abbreviations: ACE, angiotensin-I-converting enzyme; ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; ECE, endothelin-converting enzyme; LF, lactoferrin; RAS, renin angiotensin system; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat

http://dx.doi.org/10.1016/j.jff.2015.03.039
1756-4646/

JOURNAL OF FUNCTIONAL FOODS 12 (2015) 45–54

Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/jff

An antihypertensive lactoferrin hydrolysate inhibits angiotensin I-converting enzyme, modifies expression of hypertension-related genes and enhances nitric oxide production in cultured human endothelial cells

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ARTICLE INFO

Article history:
Received 5 August 2014
Received in revised form 16 October 2014
Accepted 5 November 2014
Available online

Keywords:
Lactoferrin hydrolysate
ACE inhibition
Endothelial cells
Transcriptomic analysis
NOSTRIN
Nitric oxide

ABSTRACT

This study was aimed to explore whether an antihypertensive lactoferrin hydrolysate (LFH) can inhibit angiotensin I-converting enzyme (ACE) activity and modify the expression of genes related to hypertension in human umbilical vein endothelial cells (HUVEC). LFH induced significant inhibition of ACE activity but it did not affect ACE mRNA levels after 24 h of exposure. LFH treatment significantly affected the expression of genes encoding for proteins involved in nitric oxide pathway such as soluble guanylate cyclase 1 α3 subunit (GUCY1A3; 4.42-fold increase) and nitric oxide synthase trafficking (NOSTRIN; 2.45-fold decrease). Furthermore, expression of the PTGS2/COX-2 gene encoding prostaglandin-endoperoxide synthase 2 a key component of prostaglandin synthesis was significantly increased (2.23-fold). Moreover, NOSTRIN mRNA downregulation was consistent with reduced NOSTRIN protein expression and increased NO production observed in HUVEC. The present study reveals the complexity of the effects exerted by LFH opening avenues for the better understanding of its antihypertensive effects.

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Abbreviations: ACE, angiotensin I-converting enzyme; ACTB, β-actin; ARG2, arginase type II; AT1, angiotensin type 1 receptor; B2, kinin type 2 receptor; BCA, bicinchoninic acid method; ECE, endothelin converting enzyme; eNOS, endothelial nitric oxide synthase; Fmoc, *N*-(9-fluorenyl) methoxycarbonyl; GUCY1A3, guanylate cyclase 1, soluble, alpha 3; HUVEC, human umbilical vein endothelial cells; LF, bovine lactoferrin; LFH, lactoferrin pepsin hydrolysate; NO, nitric oxide; NOSTRIN, nitric oxide synthase trafficking; PBS, phosphate buffered saline solution; PTGS2, prostaglandin-endoperoxide synthase 2; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RASS, renin–angiotensin–aldosterone system; RPL13A, ribosomal protein L13a; SBP, systolic blood pressure; SHRs, spontaneously hypertensive rats

http://dx.doi.org/10.1016/j.jff.2014.11.002
1756-4646/