HYDROLYSATES AND PEPTIDES FROM CHICKEN FOOT PROTEINS TO MANAGE HYPERTENSION

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DOCTORAL THESIS
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FEM CONSTAR que aquest treball, titulat **Hydrolysates and peptides from chicken foot proteins to manage hypertension**, que presenta Anna Mas Capdevila per a l'obtenció del títol de doctorat, ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili i que compleix els requisits per a l'obtenció de la Menció Internacional de Doctorat.

WE STATE that the present study, entitled **Hydrolysates and peptides from chicken foot proteins to manage hypertension**, presented by Anna Mas Capdevila for the award of the degree of Doctor, has been carried out under our supervision at the Departament de Bioquímica i Biotecnologia from the Universitat Rovira i Virgili and that is eligible to apply for the International Doctoral Mention.

Tarragona, 8 Novembre 2018

Les directores de la tesi doctoral

Doctoral thesis supervisors

Dr. Begoña Muguerza Marquínez

Dr. Francisca I. Bravo Vázquez

Aquesta tesi està emmarcada dins del projecte PLAN NACIONAL: RETOS DE LA SOCIEDAD (AGL-2013-40707-R i AGL-2016-77105-R), que té com a objectiu principal el desenvolupament d'un aliment integrat per al manteniment del pes corporal i per a la prevenció del risc de patologies associades amb la obesitat com la hipertensió. Anna Mas Capdevila ha estat guardonada amb una beca pre-doctoral (2015PMF-PIPF-51) per a la realització d'aquesta tesi doctoral concedida per la Universitat Rovira i Virgili dins del programa Martí i Franqués.

This thesis is framed within the PLAN NACIONAL: RETOS DE LA SOCIEDAD (AGL-2013-40707-R and AGL-2016-77105-R) project, which aims to develop an integrated food to maintain body weight and to prevent the risk of obesity related pathologies such as hypertension. Anna Mas Capdevila is the recipient of a pre-doctoral grant (2015PMF-PIPF-51) to carry out this doctoral thesis awarded by Universitat Rovira i Virgili within the Martí i Franqués program.

Als de casa

..que tot està per fer i tot és possible.

Miquel Martí i Pol

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RESUM

La hipertensió arterial es considera un dels problemes de salut pública més importants en la nostra societat. El tractament d'aquesta patologia es basa en una combinació de canvis en l'estil de vida i tractament farmacològic. No obstant, per aquells pacients en fases de desenvolupament de la malaltia i que encara no necessiten tractament farmacològic, l'ús de nutracèutics o aliments funcionals amb propietats antihipertensives està rebent molta atenció ja que podria ser una bona estratègia per evitar el desenvolupament de la hipertensió.

En aquest sentit, aquesta tesi té com a objectiu principal l'obtenció de pèptids antihipertensius a partir de la hidròlisi de proteïnes de pota de pollastre, un subproducte de la indústria avícola. Així, mitjançant la hidròlisi de la proteïnes de pota de pollastre sota condicions d'hidròlisi optimitzades, es va obtenir un hidrolitzat, Hpp11, que mostrava efecte antihipertensiu després d'una administració aguda i crònica. L'hidrolitzat Hpp11 administrat agudament produïa l'efecte antihipertensiu mitjançant la reducció de l'activitat de l'enzim convertidor d'angiotensina, mentre que després d'una administració crònica, l'efecte antihipertensiu estava mediat per una millora en la funció endotelial. Addicionalment es van caracteritzar els pèptids presents en l'Hpp11 i dos d'ells, AVFQHNCQE i QVGPLIGRYCG van mostrar efecte antihipertensiu. En particular, el pèptid AVFQHNCQE no era absorbit i produïa el seu efecte antihipertensiu mitjançant la interacció amb receptors opioides presents en el tracte gastrointestinal. La interacció amb aquests receptors desencadenava un efecte antihipertensiu mediat pel vasodilatador òxid nítric, del qual se'n veia augmentada la seva biodisponibilitat gràcies a l'efecte

antioxidant i de millora de la funció endotelial que també mostrava el pèptid.

Els resultats d'aquesta tesi obren les portes a l'ús del l'hidrolitzat i dels pèptids antihipertensius obtinguts en nutracèutics o aliments funcionals que permetrien el control i prevenció del desenvolupament de la hipertensió.

RESUMEN

La hipertensión arterial se considera uno de los problemas de salud pública más importante de nuestra sociedad. El tratamiento de esta patología se basa en una combinación de cambios en el estilo de vida y tratamiento farmacológico. No obstante, para esos pacientes que se encuentran en fase de desarrollo de la enfermedad y que aún no requieren de tratamiento farmacológico, el uso de nutracéuticos o alimentos funcionales con propiedades antihipertensivas está recibiendo mucha atención ya que podrían ser una buena estrategia para evitar el desarrollo de la hipertensión.

En este sentido, esta tesis tiene como objetivo principal la obtención de péptidos antihipertensivos a partir de la hidrólisis de proteínas de la pata de pollo, un subproducto de la industria avícola. Así, mediante la hidrólisis de las proteínas de la pata de pollo bajo condiciones de hidrólisis optimizadas se obtuvo un hidrolizado, Hpp11, que mostró efecto antihipertensivo después de una administración aguda y crónica. El hidrolizado Hpp11 administrado agudamente producía el efecto antihipertensivo mediante la reducción de la actividad de la enzima convertidora de angiotensina, mientras que después de su administración crónica, el efecto antihipertensivo estaba mediado por una mejora en la función endotelial. Adicionalmente se caracterizaron los péptidos presentes en Hpp11 y dos de ellos AVFQHNCQE y QVGPLIGRYCG mostraron efecto antihipertensivo. En particular, el péptido AVFQHNCQE no era absorbido y producía su efecto antihipertensivo mediante la interacción con receptores opioides presentes en el tracto gastrointestinal. La interacción con estos receptores desencadenaba un efecto antihipertensivo mediado por el vasodilatador óxido nítrico, del cual se veía aumentada su

biodisponibilidad gracias al efecto antioxidante y de mejora de la función endotelial que también mostró el péptido.

Los resultados de esta tesis abren las puertas al uso del hidrolizado y de los péptidos antihipertensivos obtenidos en nutracéuticos o alimentos funcionales que permitirían el control y prevención del desarrollo de la hipertensión.

SUMMARY

Hypertension is considered one of the most important public health problems in our society. The treatment of this pathology is based on lifestyle modifications and pharmacology treatment. However, for those patients developing hypertension, whose blood pressure is not high enough to warrant pharmacology treatment, the use nutraceuticals or functional foods with antihypertensive properties have attracted considerable interest. These antihypertensive natural agents could be a good strategy to avoid the development of hypertension.

In this regard, this thesis aims to obtain antihypertensive peptides through the hydrolysis of chicken foot proteins, a by-product from poultry industries. Thus, through the hydrolysis of chicken foot proteins, it was obtained the hydrolysate Hpp11, exerting antihypertensive effect after acute and chronic administration. Hpp11 administered acutely produced its antihypertensive effect by reducing the activity of angiotensin converting enzyme, while when administered chronically its antihypertensive effect was mediated by an improvement in the endothelial function. Additionally, the peptides present in Hpp11 were characterised and two of them, AVFQHNCQE and QVGPLIGRYCG, showed antihypertensive effect. In particular, the peptide AVFQHNCQE was not absorbed and produced its antihypertensive effect through the interaction with opioid receptors from the gastrointestinal tract. The interaction with these receptors leaded to a nitric oxide-mediated antihypertensive effect. Moreover, the peptide contributed to enhance nitric oxide by exhibiting antioxidant effect and improving endothelial function.

The results of this thesis open the doors to the use of the antihypertensive hydrolysate and peptides in nutraceuticals or functional foods for the control and prevention of hypertension.

LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACE2	Angiotensin converting enzyme 2
ACEI	Angiotensin converting enzyme inhibitory
Ang	Angiotensin
Arg-1	Arginase 1
AT1	Angiotensin II type 1 receptor
AT2	Angiotensin II type 2 receptor
BK	Bradykinin
BP	Blood pressure
BW	Body weight
CAF	Cafeteria
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
COX	Cyclooxygenase
CV	Cardiovascular
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
EC	Endothelial cells
ECE	Endothelin converting enzyme
eNOS	Endothelial nitric synthase
ET	Endothelin
ET_A	Endothelin-1 receptor A
ET_B	Endothelin-1 receptor B
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide

GI	Gastrointestinal
GIT	Gastrointestinal tract
GSH	Reduced glutathione
HPLC	High performance liquid chromatography
HTN	Hypertension
Klf-2	Krupple like factor 2
L-NAME	N(ω)-monomethyl-L-arginine
MetS	Metabolic syndrome
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2^-	Superoxide
PGI_2	Prostaglandin I_2 or prostacyclin
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SD	Sprague-Dawley
SHR	Spontaneously hypertensive rats
Sirt-1	Silent information regulator factor 2 related enzyme 1
VSMC	Vascular smooth muscle cell
WKY	Wistar Kyoto

INTRODUCTION

INTRODUCTION

Hypertension (HTN) is the major modifiable risk factor for all-increased risk of cardiovascular disease (CVD) (1). *This pathology has no identifiable specific cause and is currently understood as a multifactorial disease comprising genetic, environmental and behavioural factors* (2). *In this sense, HTN usually clusters with other cardiovascular (CV) risk factors such as obesity, insulin resistance, diabetes and hyperlipidaemia* (3), *which all coexist in metabolic syndrome (MetS). The effective HTN preventive approaches include lifestyle changes, primarily weight loss, diet, and exercise, and the treatment comprises the appropriate use of pharmacological agents* (4). *In recent years, bioactive peptides from proteins have emerged as a potential alternative in the prevention and management of HTN* (5). *These bioactive peptides, administered as functional foods and nutraceuticals, have attracted considerable interest as a potential alternative treatment for prehypertensive patients, whose blood pressure (BP) is mildly high but not high enough to warrant the prescription of BP-lowering medications* (6).

1. BLOOD PRESSURE

Blood pressure (BP) is the pressure that is exerted by the blood upon the walls of the blood vessels, especially arteries, and that varies depending on the muscular efficiency of the heart, the blood volume and viscosity, the state of the vascular wall and the age and health of the individual. BP is conventionally measured by systolic blood pressure (SBP) and diastolic blood pressure (DBP) determinations. SBP is defined as the maximum BP during contraction of the ventricles

while the minimum BP is DBP, recorded just prior the next contraction. The World Health Organization- International Society of Hypertension the European Society of Hypertension and the European Society of Cardiology guidelines for adults class optimal BP as less than 120 mmHg for SBP and 80 mmHg for DBP, while recordings over 120/80 mmHg and up to 139/89 mmHg are ranged as normal to high normal BP or prehypertension, depending on the coexisting medical conditions. High or elevated BP increases the risk to suffer from a CV event (7,8). In this sense, HTN or long term high BP is defined as BP values equal or over 140/90 mmHg and is considered one of the main risk factors for CVD (Figure1) (9).

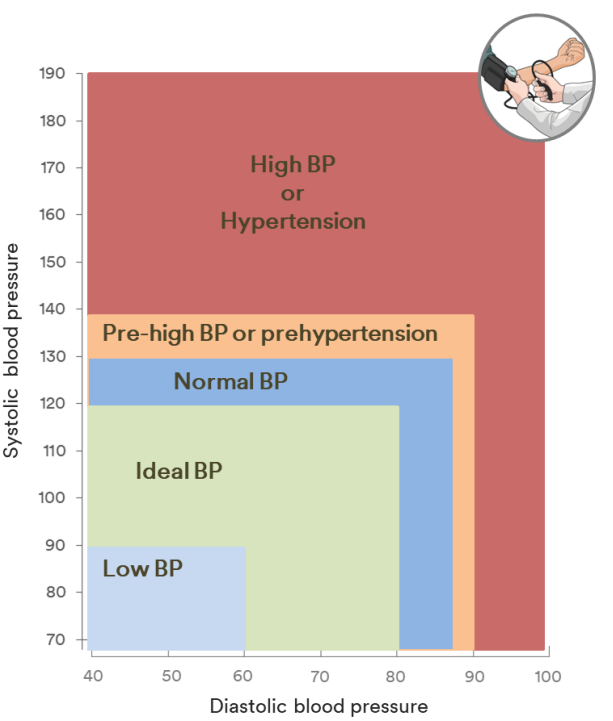

Figure 1. Classification of hypertension based in the European Society of Hypertension and the European Society of Cardiology (8).

1.1. CARDIOVASCULAR SYSTEM

The CV system conveys blood through vessels to the different parts of the body carrying nutrients and oxygen and removing carbon dioxide and other wastes. The blood is pumped by the heart into and through the vascular system to and from all parts of the body. The vascular system consists of arterial, capillary and venous components, that, as in any biologic system, their structure and function are closely related (7).

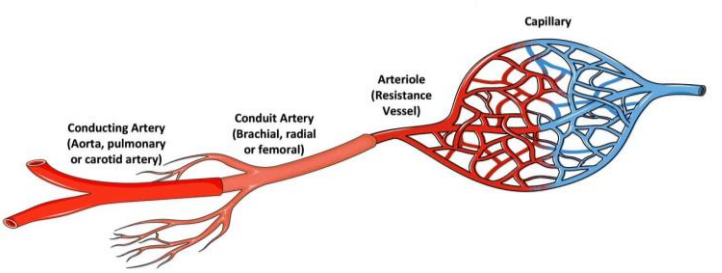

Figure 2. Schematic representation of the vascular system. Adapted from Sandoo *et al.* 2010 (10).

The blood is conducted by the conductance arteries from the heart to muscular or distribution arteries. The heart forces blood into these vessels in pulsating waves. Then, blood flows to the arterioles, or resistance small arteries, which have the distinction to contribute the most to BP and act as a control valves through which blood is released into the capillaries (Figure 2) (11). In the capillaries is where oxygen, nutrients and wastes are exchanged between the blood and the tissues. Thus, capillaries are the ultimate destination of arterial blood from the heart and the starting point for flow of venous blood back to the heart.

As mentioned above, in the CV system the heart pumps blood into the vessels in pulsating waves. In consequence, these vessels possess strong and elastic walls to guarantee fast and efficient flow to the target organs. The wall of blood vessels consists of three layers: *tunica intima*, *tunica media* and *tunica adventitia* (Figure 3).

The *tunica intima* is the most inner layer, in contact with the blood. It is the thinnest layer of the vascular wall and consists of a single sheet of endothelial cells (EC) or the endothelium covered by a surface of elastic tissues, relevant for the exchange of molecules in capillaries. The endothelium is the main structure in *tunica intima*, which not only acts as a physical barrier to separate the blood from the surrounding tissue but also participates in the regulation of coagulation, inflammation and vessel tone (12). Then it is found the *tunica media*, which consists of smooth muscle cells (VSMC) intermingled with elastic fibres. VSMC are controlled by sympathetic vasomotor nerve fibres of the autonomic nervous system and by endothelial vasodilator molecules such as nitric oxide (NO) or prostacyclin (PGI_2), and by vasoconstrictors, including endothelin-1 (ET-1) or angiotensin II (Ang II). Interestingly, the *tunica media* in conducting arteries is relatively thin and that allows the *tunica intima* to move independently from the other layers. The elastic fibres that compose *tunica media* expand with the increase of SBP and contract when the ventricles relax, pushing the blood forward. Surrounding the *tunica media*, there is an elastic layer and immediately after the *tunica adventitia*, the outermost layer, is found. The *tunica adventitia* is composed by collagenous and elastic fibres and protects the vessels from overexpansion. It is also characteristic of this layer the presence of small blood vessel, the *vasa vasorum*, which supply nutrients to the interior layers of the walls of arteries and veins. In veins, which function is to conduct blood from

the peripheral tissues to the heart, the *tunica adventitia* is the biggest layer conferring more distension to expand and contract due to changes in volume and BP (7).

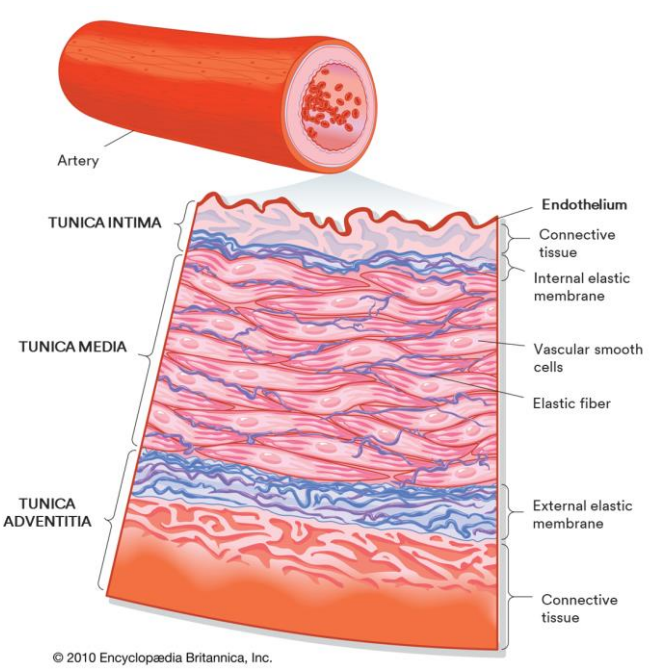

Figure 3. Transverse section of an artery. Adapted from Encyclopaedia Britannica (13).

1.2 BLOOD PRESSURE REGULATION

The maintenance of physiological BP values is crucial. While a decrease in BP could decrease blood flow and oxygen delivery to the peripheral tissues, an increase in BP could induce devastating end-organ damage (14).

BP is influenced by several parameters of the CV system and their maintenance involves a complex interaction of various elements. Among these, it is found the renin-angiotensin-aldosterone system (RAAS) and the natriuretic peptides, which induce the excretion of sodium from the kidneys regulating the vascular tone. Furthermore,

the endothelium, which release vasoactive substances controlling the vascular tone or the sympathetic nervous system, inducing Na^+ reabsorption, and the immune system which may contribute to reduce or increase BP depending on released type of inflammatory molecule (1), are demonstrated to be involved in BP control (Figure 4).

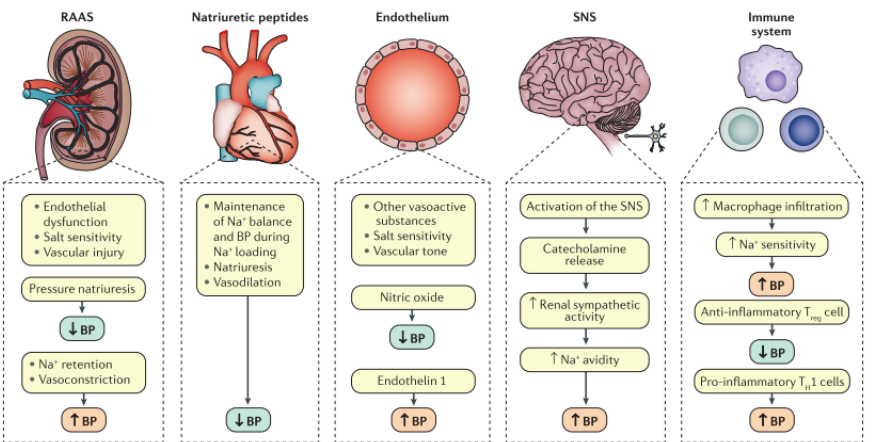

Figure 4. The major neuroendocrine systems involved in the regulation of blood pressure. Neurohumoral, immune and organ systems involved in the maintenance of blood pressure. Na^+, sodium; RAAS, renin–angiotensin–aldosterone system; SNS, sympathetic nervous system; T_{reg}, regulatory T. Extracted from Oparil *et al.* 2018 (1).

1.2.1. THE RENIN–ANGIOTENSIN–ALDOSTERONE SYSTEM

The RAAS plays a fundamental role in the homeostatic control of BP, tissue perfusion and extracellular volume. This hormonal cascade system begins with the synthesis of pro-renin (inactive) by juxtaglomerular cells in the kidney and mature renin (active) is formed by proteolytic removal of a 43-amino acid pro-segment peptide from the N-terminus of pro-renin. Renin is released from the granules of juxtaglomerular cells into the systemic circulation in response to various stimuli including changes in renal perfusion pressure, changes in delivery of NaCl, sympathetic nerve stimulation or negative

feedback by Ang II (15). Renin regulates the initial, rate-limiting step of the RAAS by cleaving the N-terminal portion of angiotensinogen (16), a protein hormone released mainly by the liver, to form angiotensin I (Ang I). Inactive decapeptide Ang I is hydrolysed by angiotensin-converting enzyme (ACE), which removes the C-terminal dipeptide His-Leu to form the octapeptide Ang II, a biologically active potent vasoconstrictor. ACE is mainly produce and found in high concentrations in the lung. However, it has also been described in kidneys, the heart, some segments of the digestive tract, the adipose tissue and the brain (17–20).

Besides of Ang I breakdown into Ang II, ACE metabolizes other peptides, including the vasodilator peptides bradykinin (BK) and kallidin, to inactive metabolites (15). Thus, the enzymatic activity of ACE results in increased vasoconstriction and decreased vasodilation.

Regarding to Ang II, it is considered the primary effector hormone of the RAAS. This hormone can act either as a systemic hormone or as locally produced factor stimulating the adrenals to produce aldosterone, which affects BP through regulating the amount of sodium by increasing or decreasing the total amount of volume in the extracellular fluid (21). In addition, Ang II contributes to the regulation of BP by influencing VSMC and sodium and volume homeostasis (16). The Ang II effects are mediated via two G-protein-coupled receptors, Ang receptor type 1 (AT1) and Ang receptor type 2 (AT2) that act in opposite directions mainly resulting in vasoconstriction when AT1 is activated and in vasodilation when Ang II binds to AT2 (22). As mentioned, the AT1 receptor has been shown to mediate most of the physiological and pathophysiological actions of Ang II, and this subtype is predominantly expressed in CV cells, such as VSMC. The

interaction of Ang II with this receptor results in the activation of a number of cytoplasmic signalling pathways, which can contribute to vasoconstriction, sodium reabsorption and aldosterone secretion in kidney and adrenal glands, sympathetic activation and cell growth, among others (23,24). On the other hand, AT2 stimulation results in vasodilation of vessels and regulates many processes implicated in vascular pathophysiology, including the inhibition of cell growth or the apoptosis of vascular cells (25). In humans, AT1 receptor is widely expressed at relatively constant levels in adults, while AT2 receptor is mainly present during foetal development and decreases rapidly after birth in most tissues (26).

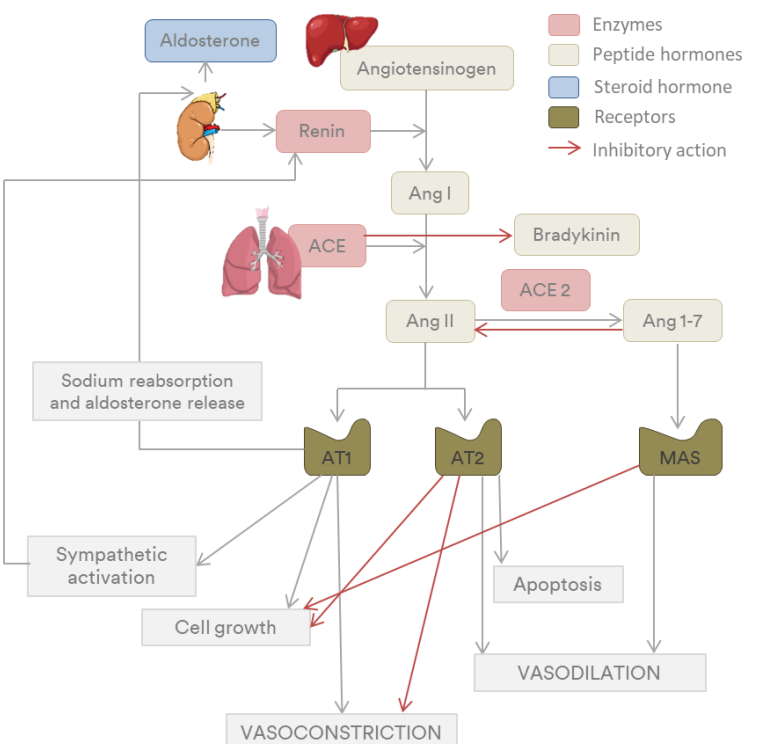

Figure 5. Schematic overview of the renin–angiotensin-aldosterone system (RAAS) and its intervening compounds. ACE: angiotensin converting enzyme; ACE2: angiotensin converting enzyme; Ang: angiotensin; AT1: Angiotensin II type 1 receptor; AT2: angiotensin II type 2 receptor; MAS: Mas receptor.

Ang II levels are also controlled by the recently identified angiotensin-converting enzyme 2 (ACE2), which is responsible for the conversion of Ang II to angiotensin (1–7), acting as a negative regulator of Ang II production. Angiotensin-(1-7), produced through hydrolysis of Ang II, interacts with the Mas receptor triggering vasodilator, anti-proliferative, anti-fibrotic, and anti-thrombotic effects (27). Thus, the balance between ACE and ACE2 is important in the regulation of Ang II levels (28).

The classical concept of RAAS is of a blood hormonal cascade, whose final product, Ang II, plays an endocrine role in the maintenance of BP and electrolyte, as well as, fluid balance (29).

1.2.2. ENDOTHELIAL FUNCTION

The endothelium is a multifunctional endocrine organ which is part of the *tunica intima,* placed between the vessel walls and the circulating blood, and has a key role in vascular homeostasis (30). The endothelium controls the fluidity and coagulation of the blood through releasing factors that regulate platelet activity, the clotting cascade, and the fibrinolytic system (31). Additionally, the endothelium has the capacity to produce cytokines and adhesion molecules that regulate the inflammatory process. However, its main action is to regulate the peripheral vasomotor tone and BP through the release of several vasodilatory factors, such as NO or PGI_2, and vasoconstrictor factors including ET-1, among others (32). In Figure 6 shows the most important endothelium-derived factors.

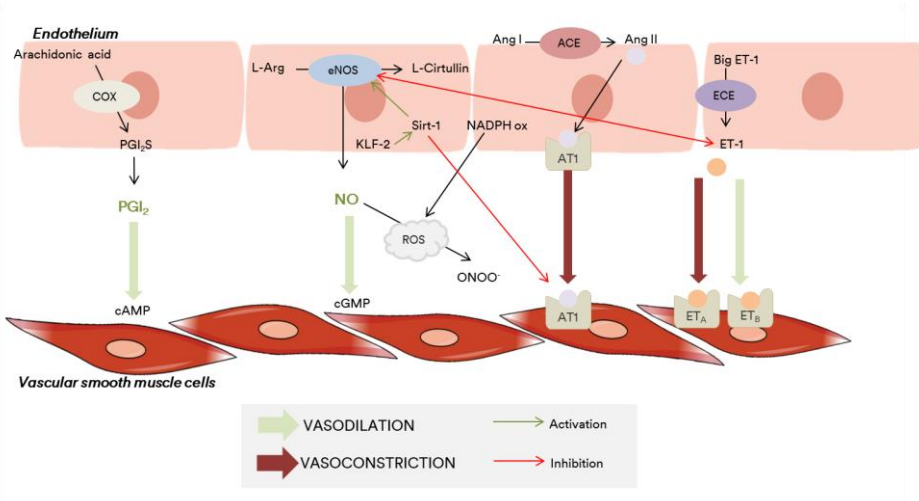

Figure 6. Endothelial-derived vasoactive factors. ACE: angiotensin converting enzyme; Ang: angiotensin; AT1: Angiotensin II type 1 receptor; AT2: Angiotensin II type 2 receptor; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; COX: cyclooxygenase; ECE: endothelin converting enzyme; EDHF: endothelial-derived hyperpolarizing factors; ET: endothelin; ET_A: endothelin receptor A; ET_B: endothelin receptor B; eNOS: endothelial nitric oxide synthase; L-Arg: L-arginine; NADPH ox: nicotinamide adenine dinucleotide phosphate-oxidase; NO: nitric oxide; PGI_2: prostaglandin I_2 or prostacyclin; PGI_2S: Prostaglandin I_2 synthase; ROS: reactive oxygen species; Sirt-1: Sirtuin-1; KLF-2, Krupple like factor 2.

1.2.2.1. ENDOTHELIAL NITRIC OXIDE

Endothelial NO is considered the most important endothelium-dependent factor which plays a key role in vasodilation, inflammation and oxidative stress (33). Moreover, NO has a number of other beneficial roles in the vessel wall, including inhibition of vascular smooth muscle proliferation, reduction of platelet aggregation, reduction of expression of adhesion molecules, inhibition of lipid oxidation and regulation of apoptosis (34).

In mammals, NO can be generated by three different isoforms of the enzyme NO synthase (NOS): neuronal NOS, inducible NOS and by

endothelial NOS (eNOS) (35). All NOs isoforms use L-arginine as the substrate and molecular oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) as co-substrates to produce NO. Flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and (6R-)5,6,7,8-tetrahydro- L-biopterin are cofactors of all isozymes (36).

Regarding eNOS, this isozyme is mainly expressed in EC, although it has also been described in cardiac myocytes, platelets, certain neurons of the brain, in syncytio-trophoblasts of the human placenta and in kidney tubular epithelial cells (37).

eNOS is synthetized as an inactive form which is bound to the protein caveolin in small invaginations of the cell membrane. Under different stimuli, including an increase in intracellular Ca^{2+}, eNOS can be activated to produce NO. Thus, rises in intracellular Ca^{2+} induce structural changes in calmodulin that allows its binding to eNOS. eNOS can be also activated by NO agonists, such as BK, acetylcholine, adenosine tri-phosphate or di-phosphate, which can influence the detachment of eNOS from caveolin by releasing Ca^{2+} from the endoplasmic reticulum (10).

As mentioned, eNOS can also be activated by other stimuli than a rise in intracellular Ca^{2+}. In this sense, the shear stress, which results from increased blood flow in the vessel, also results in the activation of eNOS. This activation is mediated by protein kinase A, which produces the activation of eNOS through the phosphorylation eNOS residue Ser-1177 (38). In addition, shear stress can also activates specialised Ca^{2+}-activated K^+ channels on the endothelial cell surface, causing K^+ efflux and Ca^{2+} influx into the cell (39).

Once eNOS is active, it transfers electrons from NADPH, via the flavins FAD and FMN, and substrate L-arginine is oxidized to L-citrulline and NO (40). The released NO diffuses to the surrounding VSMC and can act on a number of target enzymes and proteins. The most important physiological signalling pathway stimulated by NO is the activation of soluble guanylyl cyclase and the generation of cyclic guanosine monophosphate (cGMP). This molecule activates protein kinase G, the primary downstream target, which activates the myosin light-chain phosphatase. Myosin light-chain phosphatase dephosphorylates smooth muscle myosin leading to the relaxation of muscle layer and consequent vasodilatation (35,41).

Although it is appropriate to refer to eNOS as constitutive in the endothelium, maintaining basal level of eNOS protein, their expression is subjected to modest, but likely important, degrees of regulation. mRNA eNOS expression can be modulated by shear stress and by transforming growth factor-beta 1, among others (34). In this sense, deacetylase Sirtuin 1 (Sirt-1) has also been shown to regulate vascular tone via the activation of eNOS (42). Similarly, the transcription factor Krupple like factor 2 (Klf-2) is known to upregulate eNOS (43).

Regulation of NO production in the endothelium

As mentioned above, the production of NO through eNOS can be modulated by different molecular mechanisms. One of the most relevant is the Sirt-1 pathway. Sirt-1 is a nicotinamide adenine dincleotide-dependent deacetylase, highly expressed in the endothelium, involved in the regulation of aging, cell cycle regulation, apoptosis and inflammation (44). Sirt-1 plays a fundamental role in regulating endothelial NO and endothelium-dependent vascular tone

by eNOS deacetylation. In addition, many studies have demonstrated that Sirt-1 may inhibit endothelial apoptosis and improve vascular endothelial function by increasing eNOS expression, thereby exerting anti-atherosclerotic effects (45,46).

Despite the described Sirt-1 protective effects, the molecular mechanisms involved in these protective effects remain largely unknown. However, studies with resveratrol demonstrated that Klf-2 is crucial for the activation of Sirt-1. In this regard, it has been demonstrated that Klf-2 is essential in the shear stress-induced upregulation of eNOS (47). In fact, this transcription factor is considered a nuclear effector of the favourable effects of flow on endothelial gene expression and function (48).
Given the important role of Sirt-1 in maintaining vascular endothelial homeostasis, Sirt-1 and their activators have been investigated considering their potential as a target in pharmacological therapies for CVD treatment (49).

1.2.2.2. PROSTACYCLIN

Another known vasodilator factor is PGI_2 which is produced by cyclooxygenase (COX) enzyme in the EC. There are two isoforms of COX, the endothelial-constitutive COX-1 and COX-2, only expressed when the endothelium is damaged and exposed to inflammatory cytokines (50). PGI_2 is released under similar stimuli than those producing NO release. Under these stimuli, exogenous and endogenous arachidonic acid is transformed by COX to prostaglandin H_2, which is then transformed into PGI_2 by prostacyclin synthase (51). PGI_2 binds to the prostacyclin receptors located in VSMC and produces de activation of adenylate cyclase which induces the

synthesis of cyclic adenosine monophosphate (cAMP). This molecule activates protein kinase A triggering the relaxation of the smooth muscle in the same way as it does for NO (52).

1.2.2.3. ENDOTHELIN-1

ET-1 is a potent vasoconstrictor peptide which is expressed in EC. ET-1 is produced by endothelin converting enzyme under stimuli produced by inflammatory cells, such as interleukins and tumour necrosis factor α, among others (53). Contrary, its production is decreased in response to NO or PGI_2 (54). ET-1 mediates vasoconstriction and proliferation by interacting with G protein-coupled membrane- bound ET-1 type A (ETA) and ET-1 type B2 (ETB2) receptors on VSMC. When ET-1 binds to ETA or ETB2 receptors, smooth muscle Ca^{2+} channels open, allowing the entrance extracellular Ca^{2+} into the cell which causes vasoconstriction. Interestingly, EC also present ET-1 receptors, in this case ETB1. Activation of the ETB1 receptor on endothelium induces vasodilation by inducing eNOS. In consequence, there are a release of NO and an activation of PGI_2 synthesis (54). Thus, ET-1 effect is determined on the receptor localisation and the balance between ETA and ETB (1 and 2) receptors. Nevertheless, under physiological conditions, ET-1 main effect is vasoconstriction mediated by the ETA receptor, which is partly counteracted by ETB1 receptor-mediated NO release (55). In addition of ET-1 vasoactive effects, ET-1 also causes inflammation and VSMC proliferation in the vessels (56).

Therefore, a healthy endothelium is characterised to maintain normal vasculature tone and blood fluidity through a balance in the secretion of these endothelium-derived relaxing and contracting factors and presenting low production of pro-inflammatory factors (57,58).

1.2.3. ENDOTHELIAL DYSFUNCTION

The endothelium homeostasis can be altered under CV risk factors including smoking, aging, hypercholesterolemia, hyperglycaemia or HTN. The presence of these risk factors could result in a chronic inflammatory process, the loss of anti-thrombotic factors and the increase of vasoconstrictor and pro-thrombotic factors leading to the development of endothelial dysfunction (59). Thus, endothelial dysfunction is mainly characterized by the reduction of the bioavailability of vasodilators, particularly NO, and/or an increase in endothelium-derived contracting factors, such as ET-1. In this regard, the reduction in NO bioavailability it is known to be due to reduced eNOS protein levels, reduced eNOS enzymatic activity and the uncoupling of eNOS activity leading to enhanced production of superoxide, among others (60). In addition, in endothelial dysfunction, elevated reactive oxygen species (ROS) levels, deriving both from mitochondria and NADPH oxidase (NOX) activity, contribute to the reduction in NO bioavailability. This reduction is partly caused by the reaction of ROS with NO to form peroxynitrite, a key molecule which can initiate many pro-atherogenic events (61,62).

Moreover, it has been described that in endothelial dysfunction the individuals present an upregulation in ETB2 receptors in VSMC while ETB1 receptors on the EC are downregulated, resulting in an enhance in ET-1 mediated vasoconstriction (63).

As mentioned, increased ROS production is one of the main factors that contribute to the development of endothelial dysfunction. Multiple ROS are produced in the vascular wall, such as $O^{\bullet -2}$, H_2O_2 and $^{\bullet}OH$, being the mitochondria the major source. However, in the

endothelium, NOX are the main ROS source. These enzymes, under physiological concentrations, have important functions including host defence, post-translational processing of proteins, cellular signalling, regulation of gene expression, and cell differentiation (64). However, under pathological conditions these enzymes can cause cellular injury and death. Pathological states lead NOX to produce an excess of ROS that can injure vascular tissue by multiple mechanisms. For example, ROS can react with vasoactive substances and impair responses to vasodilators (65).

ROS generation is generally a cascade of reactions that starts with the production of the free radical superoxide anion ($O_2^{\bullet}$) which is the responsible of the formation of other reactive species. In the endothelium, NOX catalyse the reduction of molecular oxygen to $O_2^{\bullet}$ by using NADPH as electron donor (64). In particular, from the five NOX isoforms described, NOX2 and NOX4 are those producing ROS in the endothelium, being NOX4 the most abundant isoform (66). NOX4 appears to generate H_2O_2, although the primary product is probably $O_2^{\bullet -}$, which is rapidly dismutated to H_2O_2. This isoform produces moderate levels of ROS that are required for normal redox signalling. However, under pathological conditions, NOX4 can promote the formation of a pro-thrombogenic endothelial phenotype. Therefore, NOX4-mediated ROS production can contribute both to vascular homeostasis or to vascular injury depending on the intracellular conditions (67).

Although the pathological role of NOX4 still remains unclear, its implication in HTN has been demonstrated, suggesting that the

increase in Ang II could be the responsible of the overactivation of NOX4 and consequent increased ROS production (68). In this sense, Nishiyama *et al*. described significant increased NOX4 mRNA levels in spontaneously hypertensive rats (SHR) when compared to normotensive Wistar-Kyoto rats (WKY) (69).

Nevertheless, recent studies demonstrated that NOX4 could also present protective effects, possibly through NOX4-derived H_2O_2, which may act as a vasodilator in some vascular beds (66). In addition, a vasoprotective effect of NOX4 has been reported by enhancing eNOS and, consequently, NO availability (70). Thus, NOX4 has a relevant role in the endothelium due to its high level of expression and its constitutive activity in the vascular homeostasis. However, the physiological and pathophysiological roles of NOX4 are still on debate (71).

Therefore, the presence of CV risk factors impair the balance in the vasodilation and vasoconstrictors release, resulting in a dysfunctional endothelium which has pathophysiologic importance in the atherosclerotic process and in CV diseases including HTN (72).

2. HYPERTENSION

HTN or high BP (values over 140/90 mmHg) is a leading global health risk and is the most important preventable cause of heart disease and stroke. According to the latest reports, globally, 874 million adults have HTN which approximately represents that one in four adults suffer from HTN (1). Furthermore, 3.5 billion suffer from prehypertension (1). Thus, considering this epidemiological data, researchers have predicted the relative increase of 24 % in the

prevalence of HTN in developed countries and of 80 % in developing countries from 2000 to 2025 (73). Remarkably, the overall sex- and age-adjusted prevalence of HTN was higher in the European countries (44 %) than in the two North American countries (Canada and EEUU) (28 %) (74). Regarding Spain, the prevalence of HTN is high, around 40 % and HTN illness doubled the number of deaths from 2005 to 2015 (75). Interestingly, around 38 % of HTN patients have still not been diagnosed (76). In this regard, the lack of symptoms in early HTN states is often the reason why HTN patients are not aware of their condition. Indeed HTN is often referred to as the 'silent killer' (77).

This rapid increase in HTN prevalence will lead to dramatic rises in the incidence of CVD and their consequences, resulting in an overwhelmed health care system. The increase in HTN prevalence is attributed to an unprecedented increase in obesity and lifestyle changes in developed countries (78). From this prevalence, it is evident that HTN is an important public health challenge considering that their complications represent the major causes of morbidity and mortality.

HTN development is due to specific causes in a small fraction of cases (known as secondary HTN), but in around 90 % of patients, its etiology is unknown (essential HTN) (2). HTN is considered a multifactorial disease resulting from the combination of genetic, environmental, and behavioural factors. The HTN genetic predisposition in combination with environmental factors such as smoking or drinking, unhealthy diet, physical inactivity or obesity and overweight contribute to the pathogenesis of this disease's increase (79). In addition, ethnicity, age, gender or family history of high BP are considered non modifiable risk factors for essential HTN (80) (Figure 7).

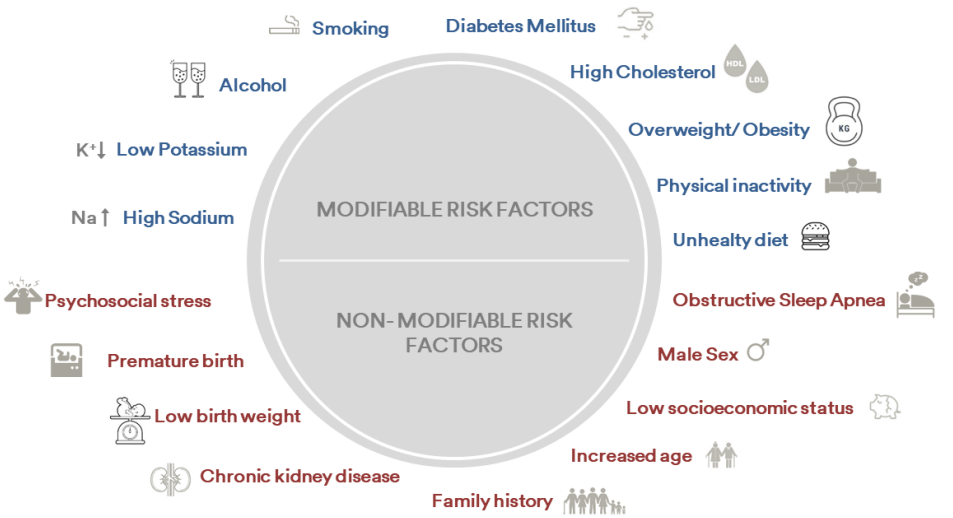

Figure 7. Representation of modifiable risk factor and non-modifiable risk factors contributing to the HTN development.

Although HTN consequences differ depending on its severity, the patient age and global medical condition, this pathology is considered chronic and in the long-term could cause end-organ damage. Thus, HTN related end-organ damage include CVDs such as stroke, accelerated coronary and systemic atherosclerosis, heart failure or chronic kidney disease which result in the most severe situations, in increased morbidity and mortality (81).

Therefore, the ultimate goal of HTN treatment is CVD risk reduction. Currently, the strategy to reduce CVD risk in HTN patient include lifestyle modifications (healthy diet and low-salt diet, exercise) and, depending on HTN stage, appropriate drug therapy (82). In addition, in diet derived-HTN or HTN associated with MetS, its treatment has demonstrated to result in a significant reduction of CV risk and to improve other MetS comorbidities (83).

2.1. HYPERTENSION ASSOCIATED TO METABOLIC SYNDROME

The MetS is defined as the simultaneous coexistence of metabolic abnormalities including obesity, glucose intolerance, dyslipidaemia, and HTN, that result in a marked increase in CV morbidity and mortality (84). In fact, High BP is considered one of the key features of MetS, and it is present in up to one third of patients with MetS (85). In this sense, it has been extensively reported that BP is strongly influenced by visceral obesity (86) and insulin resistance (87), being these two metabolic alterations described for MetS. Regarding to HTN derived from obestity, the altered production of adipocytokines in adipose tissue, the major endocrine organ in the production of these inflammatory molecules, is the main cause for the development of HTN via different pathways (88). In addition, these changes in adipocytokines release, including changes in leptin and adiponectin, have also been related to insulin resistance, which also contribute to the development of HTN (89). Indeed, insulin resistance stimulates renal sodium reabsorption (90), increases circulating ET-1 levels (91), increase leptin and non-esterified fatty acid levels, leading to the activation of the sympathetic nervous and raising BP (92,93). The activation of sympathetic nervous system induces the activation of RAAS since both systems are linked by a positive feedback relationship (94).

Additionally, it has been reported for obese animals, an upregulation of RAAS increasing the release of Ang II, suggesting that this system, crucial in BP control, is modulated by food intake (95). Moreover, in humans, RAAS is present in adipose tissue and it has been demonstrated that the production of Ang II and angiotensinogen in this tissue is increased in obese subjects (96). Furthermore, plasmatic

aldosterone has been reported to be significantly associated with the MetS-derived HTN (97).

Moreover, in rats, it has been demonstrated that both adiposity and insulin resistance are associated with the presence of systemic oxidative stress (98). Oxidative stress plays a pivotal role in the development of HTN mediating the secretion of inflammatory cytokines and inducing endothelial dysfunction (99), mainly through reducing NO bioavailability (62).

Therefore, visceral obesity, insulin resistance, oxidative stress, endothelial dysfunction, activated RAAS or increased inflammatory mediators have been proposed to be possible factors to develop HTN in MetS. All the mechanisms involved in HTN in MetS are simplified in Figure 8.

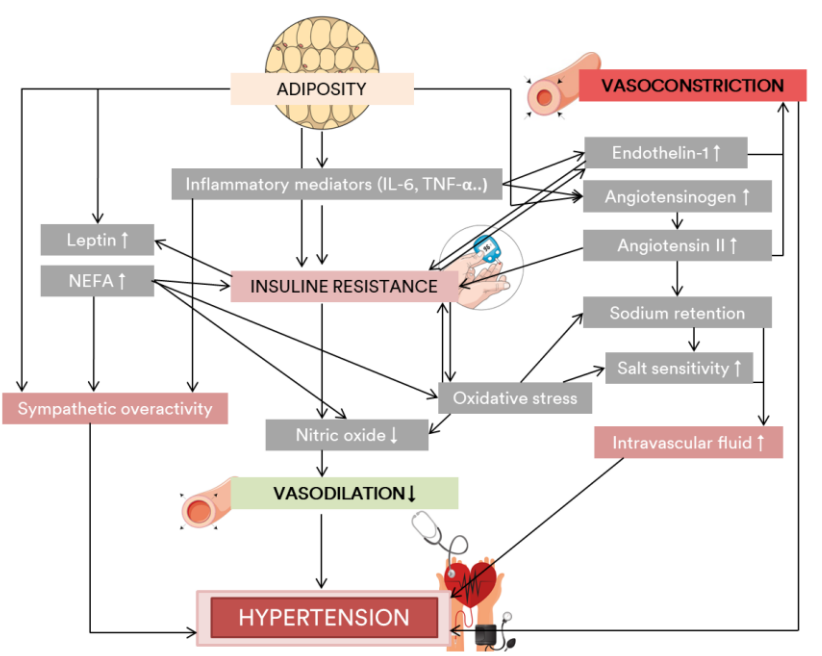

Figure 8. Proposed clinical features leading to the development of HTN in MetS. Adapted from Yanai *et al.* 2008 (100).

2.2. HYPERTENSION TREATMENT

HTN is a leading risk factor for disability and death from CV causes, thus, prevention strategies for slowing the progression of BP elevation and management strategies for lowering BP are crucial for HTN patients (101). In this sense, it has been reported that a reduction of 5 mmHg for DBP and 10 mmHg for SBP significantly decreased the risk of all major fatal and non-fatal cardiovascular outcomes (102,103). Nevertheless, BP lowering target should be determined according to the patients' global risk and accompanied diseases (104).

The treatment of HTN includes non-pharmacologic and pharmacologic approaches. For those patients presenting prehypertension and presenting less than 10 % of pre-existing CV risk, the implementation of lifestyle modifications is the most recurrent method to treat HTN (105). For those, presenting HTN or, a pre-existing CV risk of 10% or higher, both lifestyle change and medication are recommended (106).

2.2.1. NON-PHARMACOLOGICAL INTERVENTIONS

Among non-pharmacological lifestyle interventions, dietary changes, low salt diet, weight loss, increased physical activity and alcohol restriction, have been shown to reduce BP in several clinical studies (107–109). Each of these strategies are likely to reduce SBP by 3 to 8 mm Hg and DBP by 1 to 4 mmHg and these reductions have been associated with an important reduction in mortality caused by stroke and coronary heart disease (110).

Although the beneficial effects of non-pharmacological measures on BP have been well documented, it should be remarked that their BP-lowering effects are lower compared to drug therapy; therefore, these measures are suitable for those patients presenting prehypertension

or in patients with severe HTN to enhance the effects of the pharmacological interventions (111).

2.2.2. PHARMACOLOGICAL INTERVENTIONS

Extensive evidences have demonstrated the effect of pharmacological interventions in HTN treatment reducing the elevated incidence of cardiovascular morbid and fatal events related to high BP (109). The antihypertensive pharmacotherapy includes mainly five classes of drugs according their targets: diuretics, beta-blockers, calcium-channel blockers, angiotensin II receptor blockers (also known as sartans) and ACE inhibitors (112).

The diuretic therapy increases the excretion of water by inhibiting the reabsorption of sodium at different segments of the renal tubular system (113). There are three main classes of diuretics, thiazides, loop diuretics and carbonic anhydrase inhibitors (114). This therapy is effective in reducing the risk of stroke, coronary heart disease and total mortality evens at low-dose (81), due to their ability to reduce blood volume, cardiac output, and with long-term therapy, systemic vascular resistance (115). Some of the most popular diuretic drugs are furosemide (loop diuretic) or hydrochlorothiazide (thiazide), among others.

Regarding to the beta blockers, also known as beta-adrenergic blocking agents, these are competitive antagonists that block the receptors for the catecholamines epinephrine and noreprinephrine in the sympathetic nervous system (116), resulting in a reduction in cardiac output. Thus, these antihypertensive drugs are specially prescribed to reduce CV complications in patients with heart failure (117) and several studies recent have questioned the usefulness of beta-blockers as the primary tools to treat HTN (118). Some authors reported

that large evidences for beta-blockers in HTN treatment are based only in Atenolol effects, popular beta-blocker drug (119).

Another popular pharmacological therapy to ameliorate HTN is the use of calcium channel blockers. Calcium stimulates the heart to contract more forcefully and its limitation through calcium channel blockers allow blood vessels to relax and as a result, to lower BP (120,121). In addition, calcium channel blockers, including Amlodipine, are also used for treating certain types of abnormally rapid heart rhythms (122).

Besides of the use of blockers for beta-receptors and for calcium channels, antagonists for Ang II are also extensively prescribed for HTN treatment. The Ang II receptor antagonists (ARBs), including Losartan or Atacand, are the most selective blockers of the RAAS currently available (116). ARBs bind selectively block the angiotensin AT1 receptors, thereby blocking vasoconstriction and other actions typically exhibited by Ang II. Regarding the RAAS system, since 2007 it was commercialized Aliskiren, the first antihypertensive drug that produce its BP-lowering effect through the inhibition of renin. In addition, it appears that Aliskiren is the first of a new class of agents that may prove useful in the management of patients with nephropathy, heart failure and atherosclerosis in addition to HTN (123). Nevertheless, to date, the most used and successful antihypertensive drugs reducing morbidity and mortality amongst patients with HTN are the ARBs and the ACE inhibitors (124).

2.2.2.1. ACE INHIBITORS

Among medications that inhibit components of the RAAS including renin inhibitors, ARBs and ACE inhibitors, these last are considered first-line antihypertensive drugs (15). The ACE inhibitors are a heterogeneous group, both in terms of chemical structure and

pharmacokinetics, but they have in common the ability to bind competitively with the active binding site of ACE (125). Therefore, the inhibition of ACE by these drugs reduces the production of Ang II (vasoconstrictor) and increase BK (vasodilator) levels obtaining a reduction in BP.

The first ACE inhibitor, teprotide, was a nonapeptide derived from snake venom and it was identified by Ferreira *et al.* in 1965 (126). Nowadays, synthetic derivatives of this ACE inhibitor such as captopril, enalapril, lisinopril, ramipril or alacepril are used in the treatment of HTN in humans (127,128). Although these drugs show a remarkable effect in treating HTN, they can also present adverse side effects in some patients, such as dried cough, headache, fever, renal impairment, taste disturbances, skin rashes, insomnia, and angioneurotic edema allergic reactions (129).

Nevertheless, as previously mentioned, the number of prehypertensive individuals is still higher than the number of HTN individuals (1). Therefore, in order to prevent the development of HTN, it is important to act at the prehypertensive stage. In this sense, it has been demonstrated that beta blockers and diuretics, are not suitable for use in prehypertensive patients (3). Indeed, the antihypertensive drugs that have demonstrated to potentially fulfil the requirements for prehypertensive individuals are the ARBs and some calcium channel blocker drugs (3).

However, in most cases the diagnosed prehypertensive patients are not pharmacology treated and life style modifications, including low-salt diet, weight loss or increased exercise, are the strategies followed to decrease BP (130). In addition, the use of natural products found in food which inhibit ACE and lower BP have emerged as an alternative

therapy for the prevention of HTN development, especially in those prehypertensive subjects (131). Indeed, the concept of viewing food as a remedy is an ancient concept considering the words of Hippocrates who, 2500 years ago, stated: “Let food be your medicine and medicine be your food.” In consequence, the approach to the food components as potential preventive agents in HTN is nowadays considered a hot topic.

Some natural compounds including polyphenols (132) have demonstrated to reduce BP and are considered a promising alternative in HTN prevention and treatment. In this sense, the use of bioactive peptides derived from food proteins has attracted attention considering their potential as ACE inhibitors and as antihypertensive compounds (133). Thus, the scientific community and the food industry have incentivized the development of new functional foods or nutraceuticals based on these peptides to alleviate or decrease the risk to develop HTN in prehypertensive subjects (134).

3. BIOACTIVE PEPTIDES FOR THE MANAGEMENT OF HYPERTENSION

Bioactive peptides, including antihypertensive peptides, are defined as bioactive protein fragments comprised in dietary matrixes that beyond the nutritional value, they can affect biological processes or substrates and, as a result, have an impact on body function or on health conditions (135). Nevertheless, since their increasing relevance in food research and in pharmacological field, it has been defined that to be considered bioactive, peptides should produce a measurable biological effect at a physiologically realistic level and this effect should be beneficial for health, excluding from this group those

requiring high doses and those exerting potential damaging effects (136).

Bioactive peptides are formed by 3 to 20 amino acids residues (137) joined by covalent bonds also known as amide or peptide bonds. Their amino acid composition and sequence determine the activity of these active peptides once they are released from the precursor protein where they are encrypted. These bioactive peptides are usually less potent in their health beneficial effect than synthetic pharmaceutical drugs, but they are also less likely to accumulate in body tissues or to confer serious side effects because these are easily metabolized and absorbed or excreted (136). Bioactive peptides have shown numerous activities including opiate, anti-thrombotic, anti-oxidative, immunomodulatory, mineral binding, ileum contracting anti-cariogenic, anti-lipemic, osteoprotective and as mentioned, antihypertensive (138–140).

However, considering that CVD and HTN are considered important health public problems of civilization, the research focused in the obtainment of antihypertensive peptides has been receiving great interest during the past 30 years (134,141–144). In fact, more than 1700 different antihypertensive peptides have been reported in the AHTPDB "Database of Antihypertensive Peptides" database (134).

3.1. ANTIHYPERTENSIVE PEPTIDES

Since Maruyama and Suzuki in 1982 (145) reported the production of ACE inhibitory peptides by tryptic hydrolysis of casein, a great number of ACE inhibitory peptides have already been identified from dairy products proteins (146). Nevertheless, considering the potential antihypertensive effect of milk-derived peptides, being even commercialized as BP-lowering compounds (147–149), protein sources

other than dairy have also been explored and used for the obtainment of potential antihypertensive peptides (150). In this sense, animal derived-proteins from blood, meat, eggs (151,152) or plant derived-proteins from wheat, maize, soy, rice or pumpkin have been demonstrated to be a source of bioactive peptides (153,154). In this regard, protein-rich by-products generated by agro-industries have emerged as an alternative to obtain compounds with bioactivity, mainly as protein hydrolysates (155). These by-products apart from creating potential environmental problems, represent a loss of raw materials and energy, thus requiring significant investments in treatments for pollution control (156). Therefore, their alternative use to obtain bioactive peptides with a significant added-value and/or a strong economic potential is of great interest.

Specifically, the most used by-products are marine products. However in recent years, the use of chicken by-products have arisen as a potential source of bioactive peptides (157). The use of chicken by-products to produce bioactive peptides with an added-value is considered a great strategy for poultry industries, allowing to reduce by-product management. Indeed, the production and consumption of poultry products have been on the increase globally. In fact, according to Food and Agricultural Organization (FAO) (158), poultry meat production increased from 13 % in the mid-1960s to 28 % in 2018. Per capita consumption increased more than threefold over the same period being the most consumed meat in developed countries. With this massive production, thousands of tons of organic by-products in the form of viscera, head, bones, blood, feathers and feet are generated (159) representing an environmental and economic problem for the industries.

Thus, some studies on chicken have focused on extraction, isolation and utilization of their proteins. Indeed, it has been reported that chicken by-products are a good source of antihypertensive peptides (160–162). Some authors indicated that chicken by-products, including skin and leg bones, are a source of antihypertensive peptides (163)(164). Interestingly, to date, the use of chicken feet has never been used before to obtain antihypertensive peptides.

Table 1 details the most relevant protein sources used since the early 2000s for the production of peptides with ACE inhibitory activity that have demonstrated antihypertensive effects in SHR.

Source	Sequence	ACEI (IC_{50} - µM)	Antihypertensive activity (mm hg)	Dose (mg/kg)	Reference
Cuttlefish	VELYP	5.22	-20.0	10	(165)
Tuna	GDLGKTT TVSNWSP PKYKDTP	11.28	-21.0	10	(166)
Jellyfish	n.i.	1.28*	-23.0	200-800	(167)
Cod	n.i.	0.10-0.15*	-40.0	30	(168)
Oyster	VVYPWT QRF	66	- (10.0-15.0)	20	(169)
Bovine lactoferrin	DPYKLRP LPR	30.5 0.35	- (25.0-30.0)	10	(170)
Fermented milk	LHLPLP	5.5	-28.5	2	(171)

3.1.1. OBTAINING OF ANTIHYPERTENSIVE PEPTIDES

The obtainment of antihypertensive peptides usually starts with the treatment of the sample in order to solubilize the protein and thus, to facilitate enzyme accessibility to the protein. As previously

mentioned, bioactive peptides are inactive within the parent protein and therefore they require to be released from their precursor proteins to exhibit activities. In order to release the peptides from the proteins, samples are subjected to protein hydrolysis which can be performed following different methods, being the most popular, the microbial fermentation (172) and enzymatic hydrolysis (173).

Microbial fermentation is extensively used by the dairy industry, as the peptides and amino acids degraded from milk proteins during fermentation contribute to the typical flavor, aroma and texture of the products. However, as a result of microbial fermentations, bioactive peptide can also be released. In this sense, the fermentation of dairy products by *Lactobacillus helveticus* has been demonstrated to be a successful strategy for the obtaining of antihypertensive peptides (174). In a similar way, other lactic bacteria have demonstrated to produce antihypertensive peptides from dairy products (175).

As mentioned, another method extensively used to obtain protein hydrolysates or bioactive peptides is the enzymatic hydrolysis. A large number of studies have demonstrated the release of antihypertensive peptides from food proteins, by hydrolysis using different food-grade hydrolytic enzymes (individually or in combination) (176,177). In the industry the hydrolytic enzymes flavourzyme, pepsin, alcalase, chymotrypsin, trypsin or protamex have been used for the production of bioactive peptides (178).

Nevertheless, other factors than the used hydrolytic enzyme could interfere in this proteolytic procedure. In this sense, the type of protein pretreatment prior hydrolysis and the followed hydrolysis conditions (time, pH, ratio enzyme/substrate), will determine the release of bioactive peptides from protein matrixes. Thus, the optimization of the

hydrolysis conditions is an important strategy for the obtainment of peptides presenting specific activities (179,180), however, few studies obtaining bioactive peptides have reported the optimization in the hydrolysis conditions.

Once the protein hydrolysates are obtained they are screened *in vitro* for the specific activity. In this regard, one of the most popular strategies to obtain antihypertensive natural compounds is by the determination of the ACE inhibitory (ACEI) activity. ACEI measurement is considered a useful and economic methodology to select potential natural compounds able to reduce BP considering that the inhibition of this enzyme is also the target for one of the most used antihypertensive drugs, the ACE inhibitors (181).

Then, to proceed to identify ACEI peptides, the hydrolysates are subjected to ultrafiltration and/or fractionation by hydrophobicity and molecular weight mainly using reversed phase (RP) high performance liquid chromatography (HPLC) procedures. The *in vitro* ACE inhibitory activity is also tested for each fraction obtained to identify the location of the bioactive peptides derived from the enzymatic hydrolysis of the parent protein. In most instances, a single round of RP-HPLC may not be adequate to produce homogeneous peptide fractions; therefore, the most active fraction is usually subjected to a second or third round of RP-HPLC separation to obtain pure peptides. The obtained hydrolysate fraction showing the highest ACEI activity, usually the <3000 Da fraction, considering that ACEI peptides are those short in sequence, is studied by HPLC-MS to identify the sequences of the contained peptides (131). The identified peptides need to be synthetized and are required to be subjected to ACEI *in vitro* screening to distinguish those potential antihypertensive peptides. Schematic

representation of the process to obtain bioactive antihypertensive peptides is explained in Figure 9.

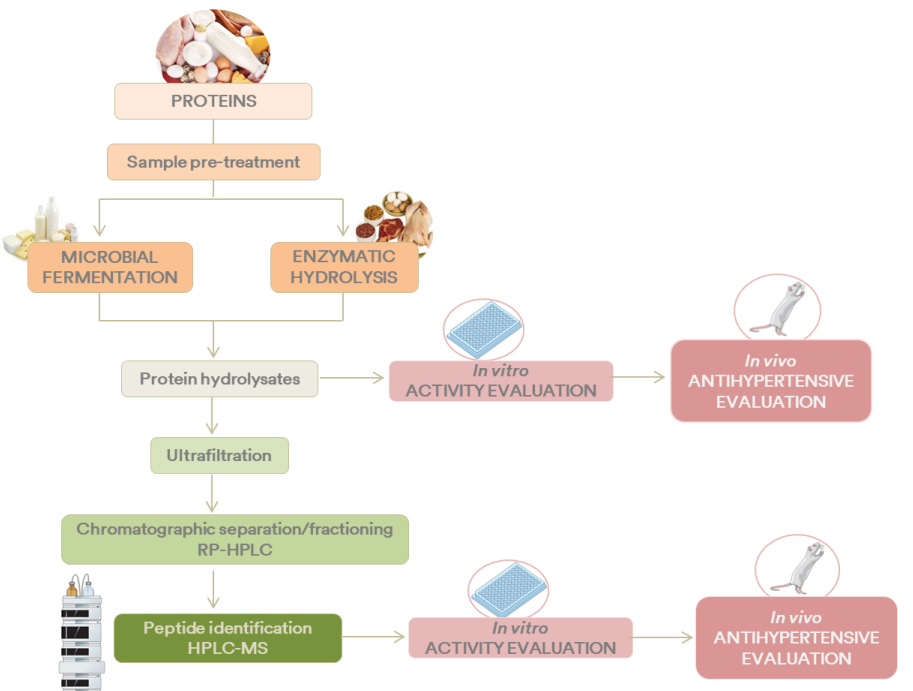

Figure 9. Schematic representation of the production process to obtain antihypertensive peptides.

Therefore, the explained procedure results in the obtainment of ACEI protein hydrolysates or peptides. Nevertheless, it is important to mention that high ACE inhibition *in vitro* does not always correspond to the same bioactivity *in vivo*. Thus, their evaluation in an animal model of HTN is required to confirm their antihypertensive properties.

3.1.2. ANIMAL MODELS TO STUDY HYPERTENSION

Historically, the SHR have been the most used animal model for testing the *in vivo* antihypertensive effects of food protein-derived protein hydrolysates and peptides. However, other animal models have been reported to be useful for the study of HTN.

3.1.2.1. SPONTANEOUSLY HYPERTENSIVE RATS

The SHR are a well-known establish genetic model to study HTN. This model was developed by Okamoto and Aoki by inbreeding Wistar rats with the highest BP values (182). This model of HTN is very similar to the HTN found in humans, developing many HTN-derived features such as cardiac hypertrophy, cardiac failure and renal dysfunction. Nevertheless, they do not present important vascular problems; despite of depressed endothelial dependent relaxations, they do not have a tendency to develop stroke, atherosclerosis or vascular thrombosis (183).

This animal model is widely used since it requires no surgery or pharmacological agent to induce HTN, and it is sensitive to most of the clinically active antihypertensive drugs, such as Captopril (184).

In this sense, in studies evaluating potential antihypertensive compounds in SHR, WKY rats, the normotensive controls of SHR, being both animal models genetically related, are included to discard possible undesirable hypotensive effects (185)

3.1.2.2. CAFETERIA-DIET FED RATS AS AN ANIMAL MODEL TO STUDY MetS

Considering that HTN frequently coexists with other CV risk factors and obesity is a major cause of HTN (186), cafeteria(CAF)-diet fed rats are considered a useful model to study diet-induced HTN. CAF-diet fed rats are a robust animal model to study MetS (187). This experimental animal model reflects the harmful effects of typical highly palatable, energy dense and unhealthy Western diet. The consumption of this diet, known as CAF diet, leads to development of MetS promoting the voluntary hyperphagia that results in rapid weight gain, impaired glucose and lipid homeostasis and HTN (187,188). In

particular, it was demonstrated by Pons *et al*. that after 6-8 weeks of CAF diet rats presented obesity and diet-induced HTN (189), similarly that what happens in humans (186). Moreover, CAF-diet fed rats also present alterations in CV system including cardiac hypertrophy, ventricular dilatation, cardiac inflammation, fibrosis, endothelial dysfunction and impairment of renal system (190).

This animal model consists on Wistar rats fed standard (STD) chow diet and water, while concurrently offered CAF diet *ad libitum*. CAF diet consists in biscuits with paté, biscuits with cheese, semi cured cheese, bacon, ensaïmada (sweetened pastry), carrots and milk with sucrose 20% (w/v). In general the composition of the CAF diet is 35% fat, 51% carbohydrates, 14% protein and 0.2% Na, whereas STD diet is 4% fat, 76% carbohydrates, 20% protein with 0.3% Na (191). Thus, the main nutritional incomes of CAF diet derive from fat and carbohydrates while in the STD diet only comes from carbohydrates. Regarding to the limitations of this model, it has been reported that in some cases, the animals could present special preference for a CAF diet particular food (192), but this animal behavior is closer to what happens in humans.

3.1.2.3. SPRAGUE-DAWLEY RATS

Sprague-dawley (SD) rats are also used as an animal model to study diet induced MetS (193). In this sense, it was reported that diet-induced obesity in SD rats also leads to the development of mild to moderate HTN (98). In addition, this model is also used to study heart failure (194). Nevertheless, this animal model is and extensively reported in studies evaluating the vascular reactivity of many compounds in *ex vivo* experiments (195–198). SD rats are very useful in pharmacological studies to evaluate the mechanisms under the antihypertensive effect exerted by specific compound or to perform a

screening of vasodilator compounds which are considered to also be able to significantly reduce BP in hypertensive animals.

Thanks to the evaluation of many compounds using different animal models, the antihypertensive effect of these compounds can be assessed. Nevertheless, once the antihypertensive effect is confirmed, it is also important to understand the mechanisms by which this compound is able to reduce BP. In this sense, considering that some of these natural antihypertensive compounds could be included in nutraceuticals or functional foods, the EFSA, European Food Safety Authority, encourage scientists to provide data including the evidence on the mechanisms by which the food/constituent could exert the claimed effect (199).

3.1.3. MECHANISMS OF ACTION

Bioactive peptides derived from dietary matrixes exhibit antihypertensive effects through various mechanisms. Most of these antihypertensive peptides have been selected to be ACE inhibitors (131). However, given the complexity of BP regulation, it is important to mention that other mechanisms than ACE inhibition have been involved in BP control exerted by those antihypertensive peptides. In this regard, it has been demonstrated that antihypertensive bioactive peptides could reduce BP by ameliorating endothelial function, modulating sympathetic nervous system through opioid-like activity and reducing vascular inflammation (200).

3.1.3.1. ANTIHYPERTENSIVE PEPTIDES MODULATING RAAS FUNCTION

RAAS system is one of the main target systems for BP control for antihypertensive peptides. In this sense, it has been described

antihypertensive peptides targeting RAAS components such as AT1 receptor (201,202), renin (203,204) or endothelin converting enzyme (205,206) which plays a vital role in BP regulation by cleaving big endothelin to form endothelin ET-1. However, ACE has long been investigated as a major physiological target for developing antihypertensive drugs and natural products (207).

The inhibition of ACE produced by those peptides reduce BP through inhibiting the production of the vasoconstrictor Ang II and avoiding BK degradation (208). Therefore, the role of ACE inhibitors is to maintain the balance between the vasoconstrictive and salt-retentive effects of Ang-II and vasodilator effects of BK (209).

In this sense, a large number of studies in animals and/or humans have demonstrated that various food-derived peptides could significantly produce an antihypertensive effect through ACE inhibition upon either intravenous or oral administration (210–215).

These ACE inhibitory peptides are usually small peptides ranging from 3 to 12 amino acid residues (144), having hydrophobic and branched-chain amino acids in their structure (216). In this sense, it was demonstrated that the active site of ACE cannot accommodate large peptide molecules (217). In addition, it has been reported that the amino acid sequence located in the three positions close to the C-terminal end of the ACEI peptide is important for activity. In this regard, the presence of aromatic, positively-charged, and basic amino acids in these positions is important for competitive binding to the ACEI active site. The most effective ACEI peptides identified contain Tyr, Phe, Trp, and/or Pro at the C-terminal (144). However, amino acids residues required for ACE inhibition at C-terminal position can be different depending on peptide size (218). Regarding to N-terminal

position, the presence of branched aliphatic amino acids such as Gly, Val, Leu, and Ile have been demonstrated to be a good substrate to ACE (219).

However, as previously mentioned, other mechanisms than ACE inhibition can be involved in the BP-lowering effect exerted by antihypertensive peptides.

3.1.3.2. ANTIHYPERTENSIVE PEPTIDES AMELIORATING ENDOTHELIAL FUNCTION

Some bioactive peptides have demonstrated to reduce BP through exerting antioxidant effect (220,221) and improving endothelial function (222). In this sense, strong evidences indicate that oxidative stress and associated oxidative damage in endothelium are mediators in the development of HTN. Under this situation, it has been demonstrated an increased production of ROS, a reduced NO synthesis and decreased bioavailability of antioxidants (223). Thus, the antioxidant action of bioactive peptides operates mainly through the scavenging of ROS and by the inactivation of free radicals (224). In this regard, the amino acid sequence plays an important role in oxygen-radical scavenging capacity. Hydrophobicity of amino acid within sequences have been considered as key factor in peptide ability to scavenge radicals (225). Moreover, antioxidant peptides can exert their antioxidant effect by the activation of endogen antioxidant systems such as reduced glutathione (GSH) (226,227).

In addition, some antihypertensive peptides have demonstrated to improve endothelial function and thus, contribute to BP reduction. These peptides mainly improve endothelial function through

enhancing vasodilatory factors, such as NO by increasing NO bioavailability or directly inducing endothelial NO formation through activation of eNOS (228,229). In fact, some of antihypertensive peptides induce NO-mediated vasorelaxation and this seems to be the mechanism underlying the BP-reducing effect (230–232). Finally, some of these antihypertensive peptides can improve endothelial functionality through the reduction of vasoconstrictor factors including ET-1 (233).

3.1.3.3. ANTIHYPERTENSIVE PEPTIDES WITH OPIOID-LIKE ACTIVITY

Interestingly, it has been reported that some antihypertensive peptides could also present opioid properties. Opioid peptides are those which have affinity for an opiate receptor as well as opiate-like effects. Endogenous opioid peptides present the same N-terminal sequence (Try-Gly-Gly-Phe) and they are originated from three precursor proteins: proopiomelanocortin, proenkephalin and prodynorphin (234). Once these peptides bind to opioid receptors, physiological effects are triggered. In this sense, opioid peptides and multiple opioid receptors are present in brain nuclei involved in cardiovascular control (235–237). In addition to the central nervous system, endogenous opioids and opioid receptors are present in peripheral tissues, including the sympathetic ganglia, adrenal medulla, vascular endothelium and gastrointestinal (GI) tract (GIT) (238,239).

Among all the derived effects triggered by the activation of opioid receptors, the stimulation of opioid receptors in the vasculature results in NO release (238,240) and thus, induce vasodilation. Indeed, there is scientific evidence that certain antihypertensive peptides are able to reduce BP through opioid-like activity. These peptides, mainly isolated

from milk proteins, interact with opioid receptors, with concomitant release of NO, which causes vasodilatation, and so reducing BP (241,242). Hérnandez-Ledesma *et al.* suggested that the interaction with opioid receptors is probably involved with the antihypertensive effects of the antihypertensive peptide α-lactorphin, considering that α-lactorphin antihypertensive effect is abolished by the opioid receptor antagonist naloxone (243).

Therefore, these studies suggest that such opioid peptides may reduce BP through receptors expressed in the GIT, implying that no absorption is required.

Nevertheless, most of antihypertensive peptides require reaching their target organ in an active form, thus, the study of their bioavailability it is important to determine their possible physiological effects *in vivo.*

3.1.4. ANTIHYPERTENSIVE PEPTIDES BIOAVAILABILITY

The antihypertensive effect of bioactive peptides is determined by their bioavailability after the oral administration. To exert this physiological effect, bioactive peptides have to remain active during GI digestion and absorption and reach the cardiovascular system. This implies resistance to GI enzymes and brush border membrane peptidases, and absorption through the intestinal epithelium (150). In this sense, those peptides selected to exert biological effects *in vitro* could undergo fragmentation into shorter peptides that are less or more active during the digestion. Therefore, *in vitro* effect could not correlate to the same *in vivo* effect. It is therefore difficult to determine the relationship between *in vitro* ACEI activity, extensively used to select potential antihypertensive sequences, and an *in vivo* hypotensive effect.

In fact, bioactive peptides when are orally administered may be inactivated by several digestive proteinases and peptidases including pepsin in the stomach, and the pancreatic enzymes trypsin, elastase, α-chymotrypsin and carboxypeptidase A and B in the small intestine. It has been noted that certain protein/peptide structures are resistant to GI digestion such as those peptides containing Pro and Hydroxy-pro residues. In addition, glycosylated peptides have been shown to be resistant to enzyme cleavage (141). Nevertheless, it has been described that some potent ACEI peptides can be produced through hydrolysis in intestinal epithelium surface by brush border membrane peptidases (244).

In this regard, the use of a cell model, such as Caco-2, a model of intestinal barrier (245), allows to study the transport of peptides through intestinal barrier and allows to identify the possible peptide structural modifications after the GI digestion.

Peptides can be transported through epithelial cells by various means, including passive transport and transcytosis, while di- and tri-peptide are transported by a peptide transporter 1 (PepT1) (208). Some antihypertensive peptides, such as the lactotripeptides Ile-Pro-Pro and Val-Pro-Pro, are demonstrated to be resistance to GI degradation and to be absorbed intact across the brush border membrane (246).

Following the absorption of a peptide into the blood stream, it may undergo hydrolysis by serum peptidases. In this sense, it has been observed for ACE inhibitory peptides that may need to be able to resist serum peptidases hydrolysis in order to reach their target organs intact and yield their antihypertensive effect (247). However, these serum peptidases can originate potent ACE inhibitors, referred to pro-drug type inhibitors of ACE, by the hydrolysis of less potent inhibitors (248).

Figure 10 shows a schematic representation of the most representative phases in bioactive peptide digestion and absorption.

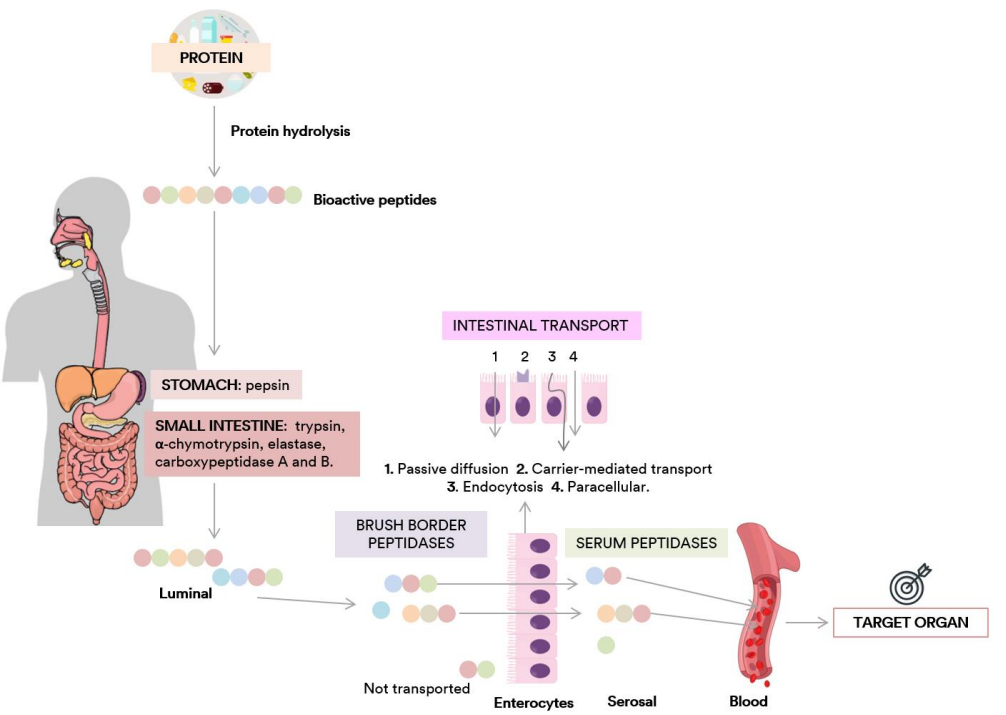

Figure 10. Digestion and transport barriers in peptide *in vivo* administration.

Nevertheless, not all the antihypertensive peptides require to be absorbed in the intestinal epithelium to induce BP-lowering effect. In this sense, the current opinion about large peptides is that the majority do not pass into the bloodstream in a significant amount and that they produce its physiological effects through the interaction with receptors on the intestinal wall (139,249). In fact, a local RAAS has been reported to be present in the small intestine, with the key components being expressed (250). In a similar way, those antihypertensive peptides that present opioid properties may lower BP through receptors expressed in the GIT, implying that no absorption is required. In this sense, the existence of three opioid receptors (μ, δ and

κ) have been reported in the small intestine, located in the myenteric plexus (251).

Thus, the bioavailability of antihypertensive peptides, especially for those inhibiting ACE, is essential for their activity. Several approaches to enhance peptide resistance to gastrointestinal digestion are used. Therefore, peptides can be modified in order to reduce the rate of enzymatic degradation and to increase bioavailability while also in some cases enhancing bioactivity (252). These modifications include end changes, glycosylation, alkylation and conformational changes.

In addition, encapsulation via nanoparticles and liposomes is also a strategy employed for antihypertensive peptides. Consequently, antihypertensive peptides may present higher therapeutic efficacy with progressive and controlled release of the drug during the degradation of the matrix (155).

3.1.5. HEALTH RELEVANCE OF ANTIHYPERTENSIVE PEPTIDES: INCORPORATION INTO FOODS PRODUCTS

Dietary antihypertensive peptides are promising components for the prevention and treatment of CVD including HTN as nutraceuticals, incorporated into foods creating functional foods or as drugs. Recently, both research centres and industries have increased their interest in the research of new sources of antihypertensive peptides, their lowering-BP effects and their bioavailability, considering their potential use as a pre-treatment for HTN.

Nevertheless, the commercialisation of antihypertensive food products depends on the availability of scientific data from *in vivo* animal and human studies positively demonstrating the BP-lowering of these products and the mechanisms involved in the BP-reduction.

Furthermore, legislation which governs health claims in relation to functional foods needs to be taken into account. In this regard, the current European Regulation on nutrition and health claims in the EU was created in 2007 (Regulation 1924/2006) and the EFSA (253) is in charge of reviewing evidence of health claims made by food companies. Interestingly, EFSA has recently approved the safety use of shrimp peptide concentrate to the control of BP in subjects with mild or moderate hypertension (254). These latest updates in EFSA food supplements approvals opening up an opportunity to develop a nutraceuticals or functional foods to BP control.

Nevertheless, some bioactive peptides are nowadays commercialised in many countries for the HTN control. Figure 10 shows a graphical representation of commercial products containing antihypertensive peptides derived from foods including Calpis®, Evolus® both containing antihypertensive peptide VPP and IPP (174), Valtyron® containing sardine isolated peptide VY (255) and PeptACE™ which include the peptide LKPNM from bonito (256).

Figure 11. Commercialized products including antihypertensive peptides.

REFERENCES

1. Oparil S, Acelajado MC, Bakris GL, Berlowitz DR, Cífková R, Dominiczak AF, et al. Hypertension. Nat Rev Dis Prim. 2018;4.
2. Bolívar JJ. Essential hypertension: An approach to its etiology and neurogenic pathophysiology. Int J Hypertens. 2013;2013.
3. Messerli FH, Williams B, Ritz E. Essential hypertension. Lancet. 2007;370(9587):591–603.
4. Cuspidi C, Tadic M, Grassi G, Mancia G. Treatment of hypertension: The ESH/ESC guidelines recommendations. Pharmacol Res. Elsevier Ltd; 2018;128:315–21.
5. Majumder K, Wu J. Molecular Targets of Antihypertensive Peptides : Understanding the Mechanisms of Action Based on the Pathophysiology of Hypertension. 2015;256–83.
6. Chen ZY, Peng C, Jiao R, Wong YM, Yang N, Huang Y. Anti-hypertensive nutraceuticals and functional foods. J Agric Food Chem. 2009;57(11):4485–99.
7. Tortora, G.; Derrickson B. Principles of anatomy and physiology. 14th ed. John Wiley and Sons, pp.523-571. 14th ed. John Wiley and Sons, editor. 2014. 523-571 p.
8. Williams B, Mancia G, Spiering W, Agabiti Rosei E, Azizi M, Burnier M, et al. 2018 ESC/ESH Guidelines for the management of arterial hypertension. European Heart Journal. 2018. 2159-2219 p.
9. World Health Organization. WHO | Hypertension [Internet]. WHO. World Health Organization; 2015. Available from: http://www.who.int
10. Sandoo A, Veldhuijzen van Zanten JJCS, Metsios GS, Carroll D, Kitas GD. The endothelium and its role in regulating vascular tone. Open Cardiovasc Med J. 2010;4(1):302–12.
11. O'Brien SF, McKendrick JD, Radomski MW, Davidge ST, Russell JC. Vascular wall reactivity in conductance and resistance arteries: Differential effects of insulin resistance. Can J Physiol Pharmacol. 1998;76(1):72–6.
12. Tunica Intima - an overview | ScienceDirect Topics.. Available from: https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/tunica-intima
13. Enxyclopaedia Britanninca. Blood vessels | anatomy |. Encyclopaedia britannica. 2010. Available from: https://www.britannica.com/science/tunica-media
14. Chalovich JM, Eisenberg E. NIH Public Access. Magn Reson Imaging. 2013;31(3):477–9.
15. Atlas S a. The renin-angiotensin aldosterone system: pathophysiological role and pharmacologic inhibition. J Manag Care Pharm. 2007;13(8 Suppl B):9–20.
16. Varagic J, Ahmad S, Nagata S, Ferrario CM. ACE2: Angiotensin II/angiotensin-(1-7) balance in cardiac and renal injury. Curr Hypertens Rep. 2014;16(3).
17. Studdy PR, Lapworth R, Bird R. Angiotensin-converting enzyme and its clinical significance--a review. J Clin Pathol [Internet]. 1983;36(8):938–47.
18. Wong MKS. Angiotensin Converting Enzymes. Handb Horm. 2016;263–5, e29-1-e29-4.
19. Perazella MA, Setaro JF. Renin-angiotensin-aldosterone system: Fundamental aspects and clinical implications in renal and cardiovascular disorders. J Nucl Cardiol. 2003;10(2):184–96.
20. Bader M, Ganten D. Update on tissue renin-angiotensin systems. J Mol Med. 2008;86(6):615–21.
21. Cachofeiro V, Miana M, de las Heras N, Martín-Fernández B, Ballesteros S, Fernández-Tresguerres J, et al. Aldosterone and the vascular system. J Steroid

Biochem Mol Biol. 2008;109(3–5):331–5.
22. Nguyen Dinh Cat A, Touyz RM. A new look at the renin–angiotensin system—Focusing on the vascular system. Peptides. 2011;32(10):2141–50.
23. Cat AND, Touyz RM. Cell signaling of angiotensin II on vascular tone: Novel mechanisms. Curr Hypertens Rep. 2011;13(2):122–8.
24. Higuchi S, Ohtsu H, Suzuki H, Shirai H, Frank GD, Eguchi S. Angiotensin II signal transduction through the AT_1 receptor: novel insights into mechanisms and pathophysiology. Clin Sci. 2007;112(8):417–28.
25. Te Riet L, Van Esch JHM, Roks AJM, Van Den Meiracker AH, Danser AHJ. Hypertension: Renin-Angiotensin-Aldosterone System Alterations. Circ Res. 2015;116(6):960–75.
26. Lemarie CA, Schiffrin EL. The angiotensin II type 2 receptor in cardiovascular disease. J Renin-Angiotensin-Aldosterone Syst. 2010;11(1):19–31.
27. Biphenyls CP. HHS Public Access. 2015;91(2):165–71.
28. Van Thiel BS, Van Der Pluijm I, Te Riet L, Essers J, Danser AHJ. The renin-angiotensin system and its involvement in vascular disease. Eur J Pharmacol. Elsevier; 2015;763:3–14.
29. Ferrario CM, Mullick AE. Renin angiotensin aldosterone inhibition in the treatment of cardiovascular disease. Pharmacol Res. Elsevier Ltd; 2017;125:57–71.
30. Khazaei M, Moien-afshari F, Laher I. Vascular endothelial function in health and diseases. Pathophysiology. 2008;15(1):49–67.
31. Van Hinsbergh VWM. Endothelium - Role in regulation of coagulation and inflammation. Semin Immunopathol. 2012;34(1):93–106.
32. Widlansky ME, Gokce N, Keaney JF, Vita JA. The clinical implications of endothelial dysfunction. J Am Coll Cardiol. Elsevier Masson SAS; 2003;42(7):1149–60.
33. Tousoulis D, Kampoli A-M, Tentolouris C, Papageorgiou N, Stefanadis C. The role of nitric oxide on endothelial function. Curr Vasc Pharmacol. 2012;10(1):4–18.
34. Searles CD. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression. Am J Cell Physiol. 2006;291:C803–16.
35. Förstermann U, Sessa WC. Nitric oxide synthases: Regulation and function. Eur Heart J. 2012;33(7):829–37.
36. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J. 2001;357(Pt 3):593–615.
37. Melikian N, Seddon MD, Casadei B, Chowienczyk PJ, Shah AM. Neuronal nitric oxide synthase and human vascular regulation. Trends Cardiovasc Med. Elsevier Inc.; 2009;19(8):256–62.
38. Fulton D, Gratton J, McCabe T, Fontana J, Fujio Y, Walsh K, et al. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature. 1999;399(6736):597–601.
39. Moens AL, Goovaerts I, Claeys MJ, Vrints CJ. Flow-mediated vasodilation: A diagnostic instrument, or an experimental tool? Chest. The American College of Chest Physicians; 2005;127(6):2254–63.
40. Vásquez-Vivar J, Kalyanaraman B, Martásek P, Hogg N, Masters BS, Karoui H, et al. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. Proc Natl Acad Sci U S A. 1998;95(16):9220–5.
41. Denninger JW, Marletta MA. Guanylate cyclase and the .NO/cGMP signaling pathway. Biochim Biophys Acta - Bioenerg. 1999;1411(2–3):334–50.
42. Gracia-Sancho J, Villarreal G, Zhang Y, Garcia-Cardena G. Activation of SIRT1 by resveratrol induces KLF2 expression conferring an endothelial vasoprotective phenotype. Cardiovasc Res. Oxford University Press; 2010 Feb 1;85(3):514–9.

43. Senbanerjee S, Lin Z, Atkins GB, Greif DM, Rao RM, Kumar A, et al. KLF2 Is a Novel Transcriptional Regulator of Endothelial Proinflammatory Activation. 2004;199(10).
44. Chalovich JM, Eisenberg E. Regulation of SIRT1 in cellular functions: role of polyphenols Sangwoon. Magn Reson Imaging. 2013;31(3):477–9.
45. Mattagajasingh I, Kim C-S, Naqvi A, Yamamori T, Hoffman TA, Jung S-B, et al. SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. Proc Natl Acad Sci. 2007 Sep 11;104(37):14855–60.
46. Zhang Q -j., Wang Z, Chen H -z., Zhou S, Zheng W, Liu G, et al. Endothelium-specific overexpression of class III deacetylase SIRT1 decreases atherosclerosis in apolipoprotein E-deficient mice. Cardiovasc Res. Oxford University Press; 2008;80(2):191–9.
47. Bhattacharya R, Senbanerjee S, Lin Z, Mir S, Hamik A, Wang P, et al. Inhibition of vascular permeability factor/vascular endothelial growth factor-mediated angiogenesis by the Kruppel-like factor KLF2. J Biol Chem. 2005;280(32):28848–51.
48. Atkins GB, Jain MK. Role of Krüppel-like transcription factors in endothelial biology. Circ Res. 2007;100(12):1686–95.
49. Wang F, Chen H-Z, Lv X, Liu D-P. SIRT1 as a Novel Potential Treatment Target for Vascular Aging and Age-Related Vascular Diseases. Curr Mol Med. 2013 Jan 1;13(1):155–64.
50. FitzGerald GA. Mechanisms of platelet activation: Thromboxane A2as an amplifying signal for other agonists. Am J Cardiol. 1991;68(7).
51. McAdam BF, Catella-Lawson F, Mardini IA, Kapoor S, Lawson JA, FitzGerald GA. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: The human pharmacology of a selective inhibitor of COX-2. Proc Natl Acad Sci. 1999;96(1):272–7.
52. Fetalvero KM, Martin KA, Hwa J. Cardioprotective prostacyclin signaling in vascular smooth muscle. Prostaglandins Other Lipid Mediat. 2007;82(1–4):109–18.
53. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature. 1988. p. 411–5.
54. Alonso D, Radomski MW. The nitric oxide-endothelin-1 connection. Heart Fail Rev. 2003;8(1):107–15.
55. Böhm F, Pernow J. The importance of endothelin-1 for vascular dysfunction in cardiovascular disease. Cardiovasc Res. 2007;76 (1); 8-18.
56. Little PJ, Ivey ME, Osman N. Endothelin-1 actions on vascular smooth muscle cell functions as a target for the prevention of atherosclerosis. Curr Vasc Pharmacol. 2008;6(3):195–203.
57. Anderson TJ, Uehata A, Gerhard MD, Meredith IT, Knab S, Delagrange D, et al. Close relation of endothelial function in the human coronary and peripheral circulations. J Am Coll Cardiol. 1995;26(5):1235–41.
58. Kinlay MB BS PS, Ganz MD P. Role of Endothelial Dysfunction in Coronary Artery Disease and Implications for Therapy. Am J Cardiol. 1997;80(9, Supplement 1):11I–16I.
59. Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: A marker of atherosclerotic risk. Arterioscler Thromb Vasc Biol. 2003;23(2):168–75.
60. Huang PL. Endothelial nitric oxide synthase and endothelial dysfunction. Curr Hypertens Rep. 2003;5(6):473–80.
61. Hsieh H, Liu C, Huang B, Tseng AHH, Wang DL. Shear-induced endothelial mechanotransduction- the interplay between reactive oxygen species (ROS) and nitric oxide (NO) and the pathophysiological implications. J Biomed Sci.

2014;1–15.
62. Incalza MA, D'Oria R, Natalicchio A, Perrini S, Laviola L, Giorgino F. Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases. Vascul Pharmacol.Elsevier; 2018;100(June 2017):1–19.
63. Masaki T, Sawamura T. Endothelin and endothelial dysfunction. Proc Japan Acad Ser B. 2006;82(1):17–24.
64. Bedard K, Krause K-H. The NOX Family of ROS-Generating NADPH Oxidases: Physiology and Pathophysiology. Physiol Rev. 2007;87(1):245–313.
65. Szocs K. Endothelial Dysfunction and Reactive Oxygen Species Production in Ischemia/Reperfusion and Nitrate Tolerance. Gen Physiol Biophys. 2004;23:265–95.
66. Ray R, Murdoch CE, Wang M, Santos CX, Zhang M, Alom-Ruiz S, et al. Endothelial Nox4 NADPH oxidase enhances vasodilatation and reduces blood pressure in vivo. Arterioscler Thromb Vasc Biol. 2011;31(6):1368–76.
67. Konior A, Schramm A, Czesnikiewicz-Guzik M, Guzik TJ. NADPH Oxidases in Vascular Pathology. Antioxid Redox Signal. 2014;20(17):2794–814.
68. Montezano AC, Touyz RM. Reactive oxygen species, vascular Noxs, and hypertension: focus on translational and clinical research. Antioxid Redox Signal. Mary Ann Liebert, Inc.; 2014;20(1):164–82.
69. Nishiyama A, Yao L, Nagai Y, Miyata K, Yoshizumi M, Kagami S, et al. Possible Contributions of Reactive Oxygen Species and Mitogen-Activated Protein Kinase to Renal Injury in Aldosterone/Salt-Induced Hypertensive Rats. Hypertension. 2004;43(4):841–8.
70. Craige SM, Chen K, Pei Y, Li C, Huang X, Chen C, et al. NADPH Oxidase 4 promotes endothelial angiogenesis through endothelial nitric oxide synthase activation. Circulation. 2011;124(6):731–40.
71. Chen F, Haigh S, Barman S, Fulton DJR. From form to function: The role of Nox4 in the cardiovascular system. Front Physiol. 2012; 1–12.
72. Endemann DH, Schiffrin EL. Endothelial dysfunction. J Am Soc Nephrol. 2004;15(8):1983–92.
73. Tu K, Chen Z, Lipscombe LL. Prevalence and incidence of hypertension from 1995 to 2005: a population-based study. Cmaj. 2008;178(11):1429–35.
74. Mensah GA. The inaugural elijah B. Saunders memorial lecture: The global consequences of hypertensi on and related dis parities. Ethn Dis. 2016;26(3):461–8.
75. Fundación española del corazón. La hipertensión mata casi el doble que hace diez años - Fundación Española del Corazón. 2017. Available from: https://fundaciondelcorazon.com
76. Menéndez E, Delgado E, Fernández-Vega F, Prieto MA, Bordiú E, Calle A, et al. Prevalencia, diagnóstico, tratamiento y control de la hipertensión arterial en España. Resultados del estudio Di@bet.es. Rev Española Cardiol. 2016;69(6):572–8.
77. Makridakis S, DiNicolantonio JJ. Hypertension: Empirical evidence and implications in 2014. Open Hear. 2014;1(1):1–8.
78. Manson JAE, Skerrett PJ, Greenland P, VanItallie TB. The Escalating Pandemics of Obesity and Sedentary Lifestyle: A Call to Action for Clinicians. Arch Intern Med. 2004;164(3):249–58.
79. Witter T, Poudevigne M, Lambrick DM, Faulkner J, Lucero AA, Page R, et al. A conceptual framework for managing modifiable risk factors for cardiovascular diseases in Fiji. Perspect Public Health. 2015;135(2):75–84.
80. American Heart Association &, American Stroke Association n.d. The Facts About High Blood Pressure | American Heart Association. 2018. Available from: http://www.heart.org/en/health-topics/high-blood-pressure/the-facts-about-

high-blood-pressure
81. Foëx P, Sear JW. Hypertension: Pathophysiology and treatment. Contin Educ Anaesthesia, Crit Care Pain. 2004;4(3):71–5.
82. Antonakoudis G, Poulimenos L, Kifnidis K, Zouras C, Antonakoudis H. Blood pressure control and cardiovascular risk reduction. 2007;114–9.
83. Redon J, Cifkova R, Laurent S, Nilsson P, Narkiewicz K, Erdine S, et al. The metabolic syndrome in hypertension: European society of hypertension position statement. J Hypertens. 2008;26(10):1891–900.
84. Kaur J. A comprehensive review on metabolic syndrome. Cardiol Res Pract. 2014;2014.
85. Schillaci G, Pirro M, Vaudo G, Gemelli F, Marchesi S, Porcellati C, et al. Prognostic value of the metabolic syndrome in essential hypertension. J Am Coll Cardiol. Elsevier Masson SAS; 2004;43(10):1817–22.
86. Sironi AM, Gastaldelli A, Mari A, Ciociaro D, Postano V, Buzzigoli E, et al. Visceral Fat in Hypertension: Influence on Insulin Resistance and -Cell Function. Hypertension. 2004;44(2):127–33.
87. Sowers JR. Type 2 diabetes mellitus and hypertension : An update. 2015;43(1):103–22.
88. Mendizábal Y, Llorens S, Nava E. Hypertension in Metabolic Syndrome : Vascular Pathophysiology. 2013;2013(Ldl).
89. Barbosa-Cortés L, López-Alarcón M, Mejía-Aranguré JM, Klünder-Klünder M, del Carmen Rodríguez-Zepeda M, Rivera-Márquez H, et al. Adipokines, insulin resistance, and adiposity as a predictors of metabolic syndrome in child survivors of lymphoma and acute lymphoblastic leukemia of a developing country. BMC Cancer. BMC Cancer; 2017;17(1):1–13.
90. Horita S, Seki G, Yamada H, Suzuki M, Koike K, Fujita T. Insulin resistance, obesity, hypertension, and renal sodium transport. Int J Hypertens. 2011;2011.
91. Sarafidis PA, Bakris GL. Review: Insulin and endothelin: An interplay contributing to hypertension development? J Clin Endocrinol Metab. 2007;92(2):379–85.
92. Sarafidis PA, Bakris GL. Non-esterified fatty acids and blood pressure elevation: A mechanism for hypertension in subjects with obesity/insulin resistance? J Hum Hypertens. 2007;21(1):12–9.
93. Anderson EA. Insulin and the sympathetic nervous system. Int J Obes Relat Metab Disord. 1993;17 Suppl 3:S86–90; discussion S97.
94. Grassi G. Renin-angiotensin-sympathetic crosstalks in hypertension: Reappraising the relevance of peripheral interactions. J Hypertens. 2001;19(10):1713–6.
95. Engeli S, Schling P, Gorzelniak K, Boschmann M, Janke J, Ailhaud G, et al. The adipose-tissue renin-angiotensin-aldosterone system: role in the metabolic syndrome? Int J Biochem Cell Biol. Elsevier; 2003;35(6):807–25.
96. Yvan-Charvet L, Quignard-Boulangé A. Role of adipose tissue renin-angiotensin system in metabolic and inflammatory diseases associated with obesity. Kidney Int. 2011;79(2):162–8.
97. Kidambi S, Kotchen JM, Grim CE, Raff H, Mao J, Singh RJ, et al. Association of adrenal steroids with hypertension and the metabolic syndrome in blacks. Hypertension. 2007;49(3 PART 2 SUPPL.):704–11.
98. Dobrian AD, Davies MJ, Schriver SD, Lauterio TJ, Prewitt RL. Oxidative Stress in a Rat Model of Obesity-Induced Hypertension. Hypertension. 2001;37(2):554–60.
99. Lobato NS, Filgueira FP, Akamine EH, Tostes RC, Carvalho MHC, Fortes ZB. Mechanisms of endothelial dysfunction in obesity-associated hypertension. Brazilian J Med Biol Res. 2012;45(5):392–400.
100. Yanai H, Tomono Y, Ito K, Furutani N, Yoshida H, Tada N. The underlying

mechanisms for development of hypertension in the metabolic syndrome. Nutr J. 2008;7(1):1–6.
101. Taler SJ. Initial Treatment of Hypertension. N Engl J Med. 2018;378(7):636–44.
102. Ettehad D, Emdin CA, Kiran A, Anderson SG, Callender T, Emberson J, et al. Blood pressure lowering for prevention of cardiovascular disease and death: A systematic review and meta-analysis. Lancet. Elsevier Ltd; 2016;387(10022):957–67.
103. Zanchetti A. Hypertension: Lower or higher blood-pressure targets for high-risk patients? Nat Rev Cardiol. Nature Publishing Group; 2016;13(11):637–8.
104. Grossman E. Blood pressure: the lower, the better: the con side. Diabetes Care. American Diabetes Association; 2011;34 Suppl 2(Suppl 2):S308-12.
105. Collier SR, Landram MJ. Treatment of prehypertension: Lifestyle and/or medication. Vasc Health Risk Manag. 2012;8(1):613–9.
106. Muntner P, Whelton PK. Using Predicted Cardiovascular Disease Risk in Conjunction With Blood Pressure to Guide Antihypertensive Medication Treatment. J Am Coll Cardiol. 2017;69(19):2446–56.
107. Gay HC, Rao SG, Vaccarino V, Ali MK. Effects of different dietary interventions on blood pressure: Systematic review and meta-analysis of randomized controlled trials. Hypertension. 2016;67(4):733–9.
108. Weiss EP, Albert SG, Reeds DN, Kress KS, Mcdaniel JL, Klein S, et al. Effects of matched weight loss from calorie restriction , exercise , or both on cardiovascular disease risk factors : a randomized intervention trial. Am Fam Physician. 2016;104(Cvd):576–86.
109. Soltani S, Shirani F, Chitsazi MJ, Salehi-Abargouei A. The effect of dietary approaches to stop hypertension (DASH) diet on weight and body composition in adults: A systematic review and meta-analysis of randomized controlled clinical trials. Obes Rev. 2016;17(5):442–54.
110. Wood JE, Barrows JG, Freis ED, Gifford RW, Kirkendall WM, Lee R, et al. Primary prevention of hypertension. J Am Osteopath Assoc. 1970;69(11):1160–2.
111. Hedayati S, Elsayed E, Reilly R. Non-pharmacological aspects of blood pressure management: what are the data? Kidney Int. 2011;79(10):1061–70.
112. James PA, Oparil S, Carter BL, Cushman WC, Dennison-Himmelfarb C, Handler J, et al. 2014 Evidence-Based Guideline for the Management of High Blood Pressure in Adults. Jama. 2014;311(5):507.
113. Wile D. Diuretics: A review. Ann Clin Biochem. 2012;49(5):419–31.
114. Shah SU, Anjum S, Littler WA. Use of diuretics in cardiovascular disease: (2) Hypertension. Postgrad Med J. 2004;80(943):271–6.
115. Silke B. Haemodynamic Impact of Diuretic Therapy in Chronic Heart Failure. 1994;84(suppl 2):115–23.
116. Frishman WH, Cheng-Lai A, Newarskas J. Current cardiovascular drugs. Current Medicine; 2005. 384 p.
117. DiNicolantonio JJ, Fares H, Niazi AK, Chatterjee S, D'Ascenzo F, Cerrato E, et al. β-Blockers in hypertension, diabetes, heart failure and acute myocardial infarction: a review of the literature. Open Hear. 2015;2(1):e000230.
118. Tomiyama H, Yamashina A. Beta-Blockers in the Management of Hypertension and/or Chronic Kidney Disease. Int J Hypertens. 2014;2014.
119. Ripley TL, Saseen JJ. β-Blockers: A Review of Their Pharmacological and Physiological Diversity in Hypertension. Ann Pharmacother. 2014;48(6):723–33.
120. The ALLHAT Officers. Major Outcomes in High-Risk Hypertensive Patients Randomized to or Calcium Channel Blocker vs Diuretic. J Am Med Assoc. 2002;288(23):2981–97.
121. Halperin AK, Cubeddu LX. The role of calcium channel blockers in the

treatment of hypertension. Am Heart J. 1986;111(2):363–82.
122. Fares H, DiNicolantonio JJ, O'Keefe JH, Lavie CJ. Amlodipine in hypertension: a first-line agent with efficacy for improving blood pressure and patient outcomes. Open Hear. BMJ Publishing Group; 2016;3(2):e000473.
123. Stanton a. Therapeutic potential of renin inhibitors in the management of cardiovascular disorders. Am J Cardiovasc Drugs. 2003;3(15):389–94.
124. Weber MA. Angiotensin II Receptor Blockers ARBs vs ACE inhibitors. Hypertension Medicine.
125. Ooi S-Y, Ball S. ACE inhibitors: their propertiesand current role in hypertension. Prescriber. 2009;20(14):15–28.
126. Ferreira SH. a Bradykinin-Potentiating Factor (Bpf) Present in the Venom of Bothrops Jararaca. Br J Pharmacol Chemother. 1965;24(1):163–9.
127. Atkinson AB, Robertson JIS. Captopril in the Treatment of Clinical Hypertension and Cardiac Failure. Lancet. 1979;314(8147):836–9.
128. Sun W, Zhang H, Guo J, Zhang X, Zhang L, Li C, et al. Comparison of the Efficacy and Safety of Different ACE Inhibitors in Patients With Chronic Heart Failure. Medicine (Baltimore). 2016;95(6):e2554.
129. Alderman CP. Angiotensin-converting enzyme inhibitors. 1994;30:55–61.
130. MacMahon S, Neal B, Rodgers A, Chobanian A, Bakris G, Black H, et al. Hypertension—time to move on. Lancet. Elsevier; 2005 Mar;365(9464):1108–9.
131. Margalef M, Bravo FI, Arola-Arnal A, Muguerza B. Natural angiotensin converting enzyme (ACE) inhibitors with antihypetensive properties. In: Andrade P., Valentao P, Pereira DM, editors. Natural products targeting clinically relevant enzymes. First. Weinheim: Wiley-VCH Verlag GmbH & Co. ; 2017. p. 45–67.
132. Rodrigo R, Gil D, Miranda-Merchak A, Kalantzidis G. Antihypertensive role of polyphenols. Adv Clin Chem. 2012;58:225–54.
133. Hayes M. Food Proteins and Bioactive Peptides: New and Novel Sources, Characterisation Strategies and Applications. Foods. 2018;7(3):38.
134. Miralles B, Amigo L, Recio I. Critical Review and Perspectives on Food-Derived Antihypertensive Peptides. J Agric Food Chem. American Chemical Society; 2018;66(36):9384–90.
135. Daliri E, Oh D, Lee B. Bioactive Peptides. Foods. 2017;6(5):32.
136. Biesalski H, Ph D, Dragsted LO, Ph D, Elmadfa I, D M, et al. Bioactive compounds : Definition and assessment of activity. 2009;25:1202–5.
137. Korhonen H, Pihlanto A. Food-derived Bioactive Peptides – Opportunities for Designing Future Foods. 2003;1297–308.
138. Möller NP, Scholz-Ahrens KE, Roos N, Schrezenmeir J. Bioactive peptides and proteins from foods: Indication for health effects. Eur J Nutr. 2008;47(4):171–82.
139. Korhonen H, Pihlanto A. Bioactive peptides: Production and functionality. Int Dairy J. 2006;16(9):945–60.
140. Li-Chan ECY. Bioactive peptides and protein hydrolysates: Research trends and challenges for application as nutraceuticals and functional food ingredients. Curr Opin Food Sci. Elsevier Ltd; 2015;1(1):28–37.
141. Norris R, Fitzgerald RJ. Antihypertensive Peptides from Food Proteins. 2013;
142. Martínez-Maqueda D, Miralles B, Recio I, Hernández-Ledesma B. Antihypertensive peptides from food proteins: a review. Food Funct. 2012;3(4):350.
143. Jao C, Huang S, Hsu K. Review article Angiotensin I-converting enzyme inhibitory peptides : Inhibition mode , bioavailability , and antihypertensive effects. BioMedicine. Elsevier Taiwan LLC; 2012;2(4):130–6.
144. Hernández-Ledesma B, Del Mar Contreras M, Recio I. Antihypertensive

peptides: Production, bioavailability and incorporation into foods. Adv Colloid Interface Sci. 2011;165(1):23–35.

145. Maruyama S, Suzuki H. A peptide inhibitor of angiotensin I converting enzyme in the tryptic hydrolysate of casein. Agric Biol Chem. Japan Society for Bioscience, Biotechnology, and Agrochemistry; 1982;46(5):1393–4.
146. Jäkälä P, Vapaatalo H. Antihypertensive Peptides from Milk Proteins. Pharmaceuticals (Basel). Multidisciplinary Digital Publishing Institute (MDPI); 2010;3(1):251–72.
147. Nakamura Y, Yamamoto N, Sakai K, Takano T. Antihypertensive Effect of Sour Milk and Peptides Isolated from It That are Inhibitors to Angiotensin I-Converting Enzyme. J Dairy Sci. 1995;78(6):1253–7.
148. Jauhiainen T, Rönnback M, Vapaatalo H, Wuolle K, Kautiainen H, Korpela R. Lactobacillus helveticus fermented milk reduces arterial stiffness in hypertensive subjects. Int Dairy J. Elsevier; 2007;17(10):1209–11.
149. Seppo L, Jauhiainen T, Poussa T, Korpela R. A fermented milk high in bioactive peptides has a blood pressure–lowering effect in hypertensive subjects. Am J Clin Nutr. 2003;77(2):326–30.
150. Aluko RE. Antihypertensive Peptides from Food Proteins. Annu Rev Food Sci Technol. 2015;6(1):235–62.
151. Bhat ZF, Kumar S, Bhat HF. Bioactive peptides of animal origin : a review. 2015;52:5377–92.
152. Stanton C. Bioactive Peptides from Muscle Sources : Meat and Fish. 2011;765–91.
153. Alimentaria A, Biochemistry F, Cieszy P. Bioinformatic-aided prediction for release possibilities of bioactive peptides from plant proteins. 2004;33(3):227–35.
154. Pal B, Vij S, Hati S. Peptides Functional significance of bioactive peptides derived from soybean. Peptides. Elsevier Inc.; 2014;54:171–9.
155. Lemes AC, Sala L, Ores C, Rafaela A, Braga C, Egea MB, et al. A Review of the Latest Advances in Encrypted Bioactive Peptides from Protein-Rich Waste.
156. Lasekan A, Abu Bakar F, Hashim D. Potential of chicken by-products as sources of useful biological resources. Waste Manag. 2013;33(3):552–65.
157. Lasekan A, Abu Bakar F, Hashim D. Potential of chicken by-products as sources of useful biological resources. Waste Manag. 2013;33(3):552–65.
158. World Agriculture: Towards 2015/2030 - An FAO perspective [Internet]. Available from: http://www.fao.org/docrep/005/y4252e/y4252e05b.htm
159. Zhu G, Zhu X, Wan X, Fan Q, Ma Y, Qian J, et al. Hydrolysis technology and kinetics of poultry waste to produce amino acids in subcritical water. J Anal Appl Pyrolysis. Elsevier B.V.; 2010;88(2):187–91.
160. Saiga A, Okumura T, Makihara T, Katsuta S, Shimizu T, Yamada R, et al. Angiotensin I-converting enzyme inhibitory peptides in a hydrolyzed chicken breast muscle extract. J Agric Food Chem. 2003;51(6):1741–5.
161. Nakade K, Kamishima R, Inoue Y, Ahhmed A, Kawahara S, Nakayama T, et al. Identification of an antihypertensive peptide derived from chicken bone extract. Anim Sci J. 2008;79(6):710–5.
162. Terashima M, Baba T, Ikemoto N, Katayama M, Morimoto T, Matsumura S. Novel angiotensin-converting enzyme (ACE) inhibitory peptides derived from boneless chicken leg meat. J Agric Food Chem. 2010;58(12):7432–6.
163. Onuh JO, Girgih AT, Malomo SA, Aluko RE, Aliani M. Kinetics of in vitro renin and angiotensin converting enzyme inhibition by chicken skin protein hydrolysates and their blood pressure lowering effects in spontaneously hypertensive rats. J Funct Foods. Elsevier Ltd; 2015;14:133–43.
164. Cheng FY, Wan TC, Liu YT, Chen CM, Lin LC, Sakata R. Determination of angiotensin-I converting enzyme inhibitory peptides in chicken leg bone

protein hydrolysate with alcalase. Anim Sci J. 2009;80(1):91–7.

165. Balti R, Bougatef A, Sila A, Guillochon D, Dhulster P, Nedjar-Arroume N. Nine novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (Sepia officinalis) muscle protein hydrolysates and antihypertensive effect of the potent active peptide in spontaneously hypertensive rats. Food Chem. 2015;170:519–25.
166. Lee S-H, Qian Z-J, Kim S-K. A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. Food Chem. 2010;118(1):96–102.
167. Liu X, Zhang M, Zhang C, Liu C. Angiotensin converting enzyme (ACE) inhibitory, antihypertensive and antihyperlipidaemic activities of protein hydrolysates from Rhopilema esculentum. Food Chem. Elsevier Ltd; 2012;134(4):2134–40.
168. Girgih AT, Nwachukwu ID, Hasan F, Fagbemi TN, Gill T, Aluko RE. Kinetics of the inhibition of renin and angiotensin I-converting enzyme by cod (Gadus morhua) protein hydrolysates and their antihypertensive effects in spontaneously hypertensive rats. Food Nutr Res. 2015;59:29788.
169. Wang J, Hu J, Cui J, Bai X, Du Y, Miyaguchi Y, et al. Purification and identification of a ACE inhibitory peptide from oyster proteins hydrolysate and the antihypertensive effect of hydrolysate in spontaneously hypertensive rats. Food Chem. 2008;111(2):302–8.
170. García-Tejedor A, Sánchez-Rivera L, Castelló-Ruiz M, Recio I, Salom JB, Manzanares P. Novel antihypertensive lactoferrin-derived peptides produced by Kluyveromyces marxianus: Gastrointestinal stability profile and in vivo angiotensin I-converting enzyme (ACE) inhibition. J Agric Food Chem. 2014 Feb;62(7):1609–16.
171. Quirós A, Ramos M, Muguerza BB, Delgado MA, Miguel M, Aleixandre A, et al. Identification of novel antihypertensive peptides in milk fermented with Enterococcus faecalis. Int Dairy J. 2007;17(1):33–41.
172. Muguerza B, Ramos M, Sánchez E, Manso MA, Miguel M, Aleixandre A, et al. Antihypertensive activity of milk fermented by Enterococcus faecalis strains isolated from raw milk. Int Dairy J. 2006;16(1):61–9.
173. Hou Y, Wu Z, Dai Z, Wang G, Wu G. Protein hydrolysates in animal nutrition: Industrial production, bioactive peptides, and functional significance. J Anim Sci Biotechnol. Journal of Animal Science and Biotechnology; 2017;8(1):1–13.
174. Nakamura Y. Studies on Anti-Hypertensive Peptides in Milk Fermented with Lactobacillus helveticus. Biosci Microflora. Japan Bifidus Foundation; 2004;23(4):131–8.
175. Beltrán-Barrientos LM, Hernández-Mendoza A, González-Córdova AF, Astiazarán-García H, Esparza-Romero J, Vallejo-Córdoba B. Mechanistic pathways underlying the antihypertensive effect of fermented milk with Lactococcus lactis NRRL b-50571 in spontaneously hypertensive rats. Nutrients. 2018;10(3).
176. Quirós A, Ramos M, Muguerza B, Delgado M a., Miguel M, Aleixandre A, et al. Identification of novel antihypertensive peptides in milk fermented with Enterococcus faecalis. Int Dairy J. 2007;17(1):33–41.
177. Pihlanto-leppa BA, Piilola K, Tupasela T, Korhonen H. Angiotensin I-converting enzyme inhibitory properties of whey protein digests : concentration and characterization of active peptides. 2018;(2000).
178. Gilamartin, L.; Jervis L. Production of Cod (Gadus morhua) Muscle Hydrolysates . Influence of Combinations of Commercial Enzyme Preparations on Hydrolysate Peptide Size Range. J Agric Food Chem. 2002;5417–23.
179. Aishah S, Amiza MA, Sarbon NM, Effendy WAM. Optimization of enzymatic protein hydrolysis conditions on Angiotensinconverting enzyme inhibitory

(ACEI) activity from blood cockle (Anadara granosa) meat. Int Food Res J. 2017;24(2):565–70.

180. Guo Y, Pan D, Tanokura M. Optimisation of hydrolysis conditions for the production of the angiotensin-I converting enzyme (ACE) inhibitory peptides from whey protein using response surface methodology. Food Chem. Elsevier Ltd; 2009;114(1):328–33.
181. Korhonen H, Pihlanto A. Bioactive peptides : Production and functionality. 2006;16:945–60.
182. Okamoto K, Aoki K. Development of a Strain of Spontaneously Hypertensive Rats. Jpn Circ J. 1963;27(3):282–93.
183. Pinto YM, Paul M, Ganten D. Lessons from rat models of hypertension. Cardiovasc Res. 1998;39(1):77–88.
184. Guillaume P, Provost D, Legrand P, Godes M, Lacroix P. Models of cardiovascular disease: Measurement of antihypertensive activity in the conscious rat (SHR, DOCA-salt, and goldblatt hypertension models). Curr Protoc Pharmacol. 2009;(SUPPL. 44):1–15.
185. Zhang-James Y, Middleton FA, Faraone S V. Genetic architecture of Wistar-Kyoto rat and spontaneously hypertensive rat substrains from different sources. Physiol Genomics. 2013;45(13):528–38.
186. Hall JE, do Carmo JM, da Silva AA, Wang Z, Hall ME. Obesity-Induced Hypertension. Circ Res. 2015 Mar 13;116(6):991–1006.
187. Sampey BP, Vanhoose AM, Winfield HM, Freemerman AJ, Muehlbauer MJ, Fueger PT, et al. Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation : Comparison to high-fat diet. Obesity. Nature Publishing Group; 2009;19(6):1109–17.
188. Coatmellec-Taglioni G, Dausse J-P, Ribiere C, Giudicelli Y. Hypertension in cafeteria-fed rats: alterations in renal 2-adrenoceptor subtypes. Am J Hypertens. Oxford University Press; 2000 May 1;13(5):529–34.
189. Pons Z, Margalef M, Bravo FI, Arola-arnal A, Muguerza B. Chronic administration of grape-seed polyphenols attenuates the development of hypertension and improves other cardiometabolic risk factors associated with the metabolic syndrome in cafeteria diet-fed rats. 2018;(2017):200–8.
190. Coatmellec-Taglioni G, Dausse JP, Ribière C, Giudicelli Y. Hypertension in cafeteria-fed rats: Alterations in renal α2-adrenoceptor subtypes. Am J Hypertens. 2000;13(5 I):529–34.
191. Pons Z, Margalef M, Bravo FI, Arola A, Muguerza B. Acute administration of single oral dose of grape seed polyphenols restores blood pressure in a rat model of metabolic syndrome : role of nitric oxide and prostacyclin. Eur J Nutr. Springer Berlin Heidelberg; 2016;749–58.
192. Moore BJ. The cafeteria diet--an inappropriate tool for studies of thermogenesis. J Nutr. 1987;117(2):227–31.
193. Wong SK, Chin K-Y, Suhaimi FH, Fairus A, Ima-Nirwana S. Animal models of metabolic syndrome: a review. 2016.
194. Hasenfuss G. Animal models of human cardiovascular disease, heart failure and hypertrophy. Cardiovasc Res. 1998;39(1):60–76.
195. Rahmani MA, DeGray G, David V, Ampy FR, Jones L. Comparison of calcium import as a function of contraction in the aortic smooth muscle of Sprague-Dawley, Wistar Kyoto and spontaneously hypertensive rats. Front Biosci. 1999;4(15):D408–15.
196. Tanaka M, Tokuyasu M, Matsui T, Matsumoto K. Endothelium-independent vasodilation effect of di- and tri-peptides in thoracic aorta of Sprague–Dawley rats. Life Sci. 2008 ;82(15–16):869–75.
197. Erez-vizca FP, Ibarra M, Cogolludo AL, Duarte J, A-arn FZ, Moreno L, et al. Endothelium-Independent Vasodilator Effects of the Flavonoid Quercetin and

Its Methylated Metabolites in Rat Conductance and Resistance Arteries. 2002;302(1):66–72.

198. Miguel M, Alvarez Y, López-Fandiño R, Alonso MJ, Salaices M. Vasodilator effects of peptides derived from egg white proteins. Regul Pept. 2007;140(3):131–5.
199. Draft Scientific and technical guidance for the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) 5. Available from: www.efsa.europa.eu/efsajournal
200. Udenigwe CC, Mohan A. Mechanisms of food protein-derived antihypertensive peptides other than ACE inhibition. J Funct Foods. Elsevier Ltd; 2014;8(1):45–52.
201. Yu Z, Yin Y, Zhao W, Chen F, Liu J. Antihypertensive effect of angiotensin-converting enzyme inhibitory peptide RVPSL on spontaneously hypertensive rats by regulating gene expression of the renin-angiotensin system. J Agric Food Chem. 2014;62(4):912–7.
202. Fernández-Musoles R, Castelló-Ruiz M, Arce C, Manzanares P, Ivorra MD, Salom JB. Antihypertensive mechanism of lactoferrin-derived peptides: Angiotensin receptor blocking effect. J Agric Food Chem. 2014;62(1):173–81.
203. Li H, Aluko RE. Identification and inhibitory properties of multifunctional peptides from pea protein hydrolysate. J Agric Food Chem. 2010;58(21):11471–6.
204. Girgih AT, Udenigwe CC, Li H, Adebiyi AP, Aluko RE. Kinetics of enzyme inhibition and antihypertensive effects of hemp seed (Cannabis sativa L.) protein hydrolysates. JAOCS, J Am Oil Chem Soc. 2011;88(11):1767–74.
205. Okitsu M, Morita A, Kakitani M, Okada M, Yokogoshi H. Inhibition of the endothelin-converting enzyme by pepsin digests of food proteins. Biosci Biotechnol Biochem. 1995;59(2):325–6.
206. Fernández-Musoles R, Salom JB, Martínez-Maqueda D, López-Díez JJ, Recio I, Manzanares P. Antihypertensive effects of lactoferrin hydrolyzates: Inhibition of angiotensin- and endothelin-converting enzymes. Food Chem. Elsevier Ltd; 2013;139(1–4):994–1000.
207. Takai S, Jin D, Sakaguchi M, Miyazaki M. Significant target organs for hypertension and cardiac hypertrophy by angiotensin-converting enzyme inhibitors. Hypertens Res. 2004;27(3):213–9.
208. Vermeirssen V, Camp J Van, Verstraete W. Bioavailability of angiotensin I converting enzyme inhibitory peptides. Br J Nutr. 2004;92(3):357.
209. Mora L, Gallego M, Toldrá F. ACEI-Inhibitory Peptides Naturally Generated in Meat and Meat Products and Their Health Relevance. Nutrients. 2018;10(9):1259.
210. Miguel M, Alonso MJ, Salaices M, Aleixandre A, López-Fandiño R. Antihypertensive, ACE-inhibitory and vasodilator properties of an egg white hydrolysate: Effect of a simulated intestinal digestion. Food Chem. Elsevier; 2007;104(1):163–8.
211. Mirdhayati I, Hermanianto J, Wijaya CH, Sajuthi D, Arihara K. Angiotensin converting enzyme (ACE) inhibitory and antihypertensive activities of protein hydrolysate from meat of Kacang goat (Capra aegagrus hircus). J Sci Food Agric. 2016;96(10):3536–42.
212. Miguel M, Manso M, Aleixandre A, Alonso MJ, Salaices M, Lopez-Fandino R. Vascular effects, angiotensin I-converting enzyme (ACE)-inhibitory activity, and anti hypertensive properties of peptides derived from egg white. J Agric Food Chem. 2007;55(26):10615–21.
213. Yoshii H, Tachi N, Ohba R, Sakamura O, Takeyama H, Itani T. Antihypertensive effect of ACE inhibitory oligopeptides from chicken egg yolks. Comp Biochem Physiol - C Pharmacol Toxicol Endocrinol. 2001;128(1):27–33.

214. Majumder K, Chakrabarti S, Morton JS, Panahi S, Kaufman S, Davidge ST, et al. Egg-derived ACE-inhibitory peptides IQW and LKP reduce blood pressure in spontaneously hypertensive rats. J Funct Foods. Elsevier Ltd; 2015;13:50–60.
215. Miguel M, Contreras MM, Recio I, Aleixandre A. ACE-inhibitory and antihypertensive properties of a bovine casein hydrolysate. Food Chem. 2009;112(1):211–4.
216. Pripp AH, Isaksson T, Stepaniak L, Sørhaug T. Quantitative structure-activity relationship modelling of ACE-inhibitory peptides derived from milk proteins. Eur Food Res Technol. 2004;219(6):579–83.
217. Natesh R, Schwager SLU, Sturrock ED, Acharya KR. Crystal structure of the human enzyme – lisinopril complex. Nature. 2003;421(1995):1427–9.
218. Wu J, Aluko RE, Nakai S. Structural requirements of angiotensin I-converting enzyme inhibitory peptides: Quantitative structure-activity relationship modeling of peptides containing 4-10 amino acid residues. QSAR Comb Sci. 2006;25(10):873–80.
219. Li GH, Le GW, Shi YH, Shrestha S. Nutrition research. [Internet]. Nutrition research. Elsevier Inc; 2004.
220. Escudero E, Aristoy MC, Nishimura H, Arihara K, Toldrá F. Antihypertensive effect and antioxidant activity of peptide fractions extracted from Spanish dry-cured ham. Meat Sci. Elsevier Ltd; 2012;91(3):306–11.
221. Girgih AT, He R, Malomo S, Offengenden M, Wu J, Aluko RE. Structural and functional characterization of hemp seed (Cannabis sativa L.) protein-derived antioxidant and antihypertensive peptides. J Funct Foods. Elsevier Ltd; 2014;6(1):384–94.
222. Chakrabarti S, Wu J. Bioactive peptides on endothelial function. Food Sci Hum Wellness [Internet]. Elsevier; 2016;5(1):1–7.
223. Silva BR, Pernomian L, Bendhack LM. Contribution of oxidative stress to endothelial dysfunction in hypertension. Front Physiol. 2012;3 DEC(December):1–5.
224. Ahmed AS, El-Bassiony T, Elmalt LM, Ibrahim HR. Identification of potent antioxidant bioactive peptides from goat milk proteins. Food Res Int. 2015;74:80–8.
225. Sarmadi BH, Ismail A. Antioxidative peptides from food proteins: A review. Peptides. Elsevier Inc.; 2010;31(10):1949–56.
226. Udenigwe CC, Girgih AT, Mohan A, Gong M, Malomo SA, Aluko RE. Antihypertensive and bovine plasma oxidation-inhibitory activities of spent hen meat protein hydrolysates. J Food Biochem. 2017;41(4):1–8.
227. Elias RJ, Kellerby SS, Decker EA. Antioxidant activity of proteins and peptides. Crit Rev Food Sci Nutr. 2008;48(5):430–41.
228. Chakrabarti S, Wu J. Bioactive peptides on endothelial function. Food Sci Hum Wellness. Beijing Academy of Food Sciences.; 2016;5(1):1–7.
229. Ballard KD, Bruno RS, Seip RL, Quann EE, Volk BM, Freidenreich DJ, et al. Acute ingestion of a novel whey-derived peptide improves vascular endothelial responses in healthy individuals: A randomized, placebo controlled trial. Nutr J. 2009;8(1):1–11.
230. Matoba N, Usui H, Fujita H, Yoshikawa M. A novel anti-hypertensive peptide derived from ovalbumin induces nitric oxide-mediated vasorelaxation in an isolated SHR mesenteric artery. FEBS Lett. 1999;452(3):181–4.
231. Miguel M, Alonso MJ, Salaices M, Aleixandre A, López-Fandiño R. Antihypertensive, ACE-inhibitory and vasodilator properties of an egg white hydrolysate: Effect of a simulated intestinal digestion. Food Chem. 2007;104(1):163–8.
232. Tatemoto K, Takayama K, Zou MX, Kumaki I, Zhang W, Kumano K, et al. The

novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. Regul Pept. 2001;99(2–3):87–92.

233. Maes W, Van Camp J, Vermeirssen V, Hemeryck M, Ketelslegers JM, Schrezenmeir J, et al. Influence of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg (ALPMHIR) on the release of endothelin-1 by endothelial cells. Regul Pept. 2004;118(1–2):105–9.
234. Hollt V. Multiple endogenous opioid peptides. Tins. 1983;(1979):28–30.
235. Reid JL, Rubin PC, Petty MA. Opioid Peptides and Central Control of Blood Pressure. Clin Exp Hypertens Part A Theory Pract. 1984;6(1–2):107–20.
236. Peptides THEO, Morphine EOF, Peptide THEO. the Opioid Peptides. 2013;1–8.
237. Tavares T, Sevilla M-Á, Montero M-J, Carrón R, Malcata FX. Acute effect of whey peptides upon blood pressure of hypertensive rats, and relationship with their angiotensin-converting enzyme inhibitory activity. Mol Nutr Food Res. 2012;56(2):316–24.
238. Hughes J, Kosterlitz HW, Smith TW. the Distribution of Methionine-Enkephalin and Leucine-Enkephalin in the Brain and Peripheral Tissues. Br J Pharmacol. 1977;61(4):639–47.
239. Cadet P, Bilfinger T V., Fimiani C, Peter D, Stefano GB. Human vascular and cardiac endothelia express mu opiate receptor transcripts. Endothel J Endothel Cell Res. 2000;7(3):185–91.
240. Wu, Q.; Wang, H.; Li, J.; Zhou, P.; Wang, Q-L.; Zhao, L.; Fan, R.; Wang, Y-M; Xu, X-Z; Yi, D-; Yu, S-Q; Pei J-M. k-Opioid Receptor Stimulation Improves Endothelial Function in Hypoxic Pulmonary Hypertension. PLoS One. 2013;8(5).
241. Ijäs H, Collin M, Finckenberg P, Pihlanto-Leppälä A, Korhonen H, Korpela R, et al. Antihypertensive opioid-like milk peptide α-lactorphin: Lack of effect on behavioural tests in mice. Int Dairy J. 2004;14(3):201–5.
242. Nurminen ML, Sipola M, Kaarto H, Pihlanto-Leppälä A, Piilola K, Korpela R, et al. Alpha-lactorphin lowers blood pressure measured by radiotelemetry in normotensive and spontaneously hypertensive rats. Life Sci. 2000;66(16):1535–43.
243. Hernández-ledesma B, Contreras M, Recio I. Antihypertensive peptides : Production , bioavailability and incorporation into foods. Adv Colloid Interface Sci. Elsevier B.V.; 2011;165(1):23–35.
244. Da A, Lasuncio MA, Quiro A. Bioavailability of the antihypertensive peptide LHLPLP : Transepithelial flux of HLPLP. 2008;18:279–86.
245. Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F. The Caco-2 cell line as a model of the intestinal barrier: in£uence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biol Toxicol. 2005;21(1):1–26.
246. Foltz M, Cerstiaens A, van Meensel A, Mols R, van der Pijl PC, Duchateau GSMJE, et al. The angiotensin converting enzyme inhibitory tripeptides Ile-Pro-Pro and Val-Pro-Pro show increasing permeabilities with increasing physiological relevance of absorption models. Peptides. 2008;29(8):1312–20.
247. Mine Y, Shahidi F. Nutraceutical proteins and peptides in health and disease. CRC/Taylor and Francis; 2006. 668 p.
248. Iroyukifujita H, Eiichiyokoyama K, Yoshikawa M. Classification and Antihypertensive Activity of Angiotensin I-Converting Enzyme Inhibitory Peptides Derived from Food Proteins. J Food Sci. 2000;65(4):564–9.
249. Miner-Williams WM, Stevens BR, Moughan PJ. Are intact peptides absorbed from the healthy gut in the adult human? Nutr Res Rev. 2014;27(2):308–29.
250. Wong TP, Debnam ES, Leung PS. Involvement of an enterocyte renin-angiotensin system in the local control of SGLT1-dependent glucose uptake across the rat small intestinal brush border membrane. J Physiol. Wiley-Blackwell; 2007 Oct 15;584(Pt 2):613–23.

251. Nishimura E, Buchan AMJ, Mcintosh CHS. Autoradiographic Localization of J-L-and 8-Type Opioid Receptors in the Gastrointestinal Tract of the Rat and Guinea Pig. Gastroenterology. 1986.
252. Adessi C, Soto C. Converting a Peptide into a Drug: Strategies to Improve Stability and Bioavailability. Curr Med Chem. 2002;9(9):963–78.
253. Nutrition and Health Claims - European Commission. Available from: https://ec.europa.eu/food/safety/labelling_nutrition/claims_en
254. Turck D, Bresson J, Burlingame B, Dean T, Fairweather-Tait S, Heinonen M, et al. Safety of shrimp peptide concentrate as a novel food pursuant to Regulation (EU) 2015/2283. EFSA J. Wiley-Blackwell; 2018 May;16(5).
255. Kawasaki T, Seki E, Osajima K, Yoshida M, Asada K, Matsui T, et al. Antihypertensive effect of valyl-tyrosine, a short chain peptide derived from sardine muscle hydrolyzate, on mild hypertensive subjects. J Hum Hypertens. 2000;14(8):519–23.
256. Fujita H, Yoshikawa M. LKPNM: A prodrug-type ACE-inhibitory peptide derived from fish protein. Immunopharmacology. 1999;44(1–2):123–7.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

In recent years it has been extensively demonstrated that dietary protein hydrolysis is a successful strategy for the obtaining of peptides presenting beneficial effects on human health. In this sense, antihypertensive peptides are of great interest since HTN is the principal risk of CVD, which in turn is the main cause of mortality in western societies. The antihypertensive effect of these peptides is mainly related to their capacity to inhibit ACE, a fundamental regulatory enzyme in the RAAS with a key role in the physiological regulation of the BP. Indeed, the selection of bioactive compounds according their ACE inhibitory activities is an important strategy in the research of new antihypertensive agents. In this regard, the use of food by-products has demonstrated to be a successful strategy for the obtainment of bioactive peptides, including ACEI peptides. Additionally, these by-product derived peptides can be used as functional ingredients with significant added-value and strong economic potential. In this sense, chicken processing industries generate large amount of by-products, including chicken feet. Therefore, **the hydrolysis of chicken foot proteins could be a good strategy to obtain ACEI peptides with antihypertensive properties after their oral administration.**

Thus, the main objective of this thesis was to obtain ACEI hydrolysates with BP-lowering effect from chicken foot proteins and identify their bioactive peptides.

In order to assess the established assumption, specific objectives were proposed:

1. **To obtain antihypertensive hydrolysates from chicken foot proteins (*Chapter 1*).**

Protein hydrolysis is required to release the potential bioactive peptides enclosed in the native proteins. However, hydrolysis conditions such as raw material treatment, pH, temperature, type of enzyme and enzyme/substrate relation determine the production of different peptides and their bioactivities. Thus, the optimization of hydrolysis conditions it is important for the obtainment of peptides with high ACEI activity, and therefore, potential antihypertensive properties. Nevertheless, since *in vitro* ACEI activity does not always correspond to the same *in vivo* activity, the evaluation of the *in vivo* antihypertensive activity is required. Considering these facts, the following aim was proposed.

a) To determine the optimal hydrolysis conditions to obtain chicken foot hydrolysates presenting high ACEI activity and antihypertensive effect in SHR **[Manuscript 1].**

2. **To stablish the most effective dose and the mechanisms involved in the BP-lowering effect of the antihypertensive chicken foot hydrolysate Hpp11 after acute administration *(Chapter 2).***

The study of the most effective dose and mechanisms involved in the effect of antihypertensive compounds is essential in the investigation of potential functional ingredients for the management of HTN. In addition, the BP-lowering effect of antihypertensive agents must be linked to the HTN state, discarding possible hypotensive effects under normotensive conditions. Thus, the following goal was proposed:

a) To determinate the most effective antihypertensive dose of the chicken foot hydrolysate Hpp11 after acute administration to SHR, establish the mechanisms underlying its antihypertensive effect and discard a possible hypotensive effect in normotensive WKY rats **[Manuscript 2].**

3. To evaluate the effect of long-term administration of chicken foot hydrolysate Hpp11 in diet-induced hypertension *(Chapter 3)*.

However, HTN is considered a chronic pathology requiring chronic treatment, which frequently occurs concurrently with many other CV risk factors related to lifestyle, such as obesity or impaired glucose tolerance, which all contribute to MetS. In this sense, CAF-diet fed rats are considered a good model for the study of human diet-induced HTN. Therefore, the following aim was proposed.

a) To investigate the effect of long-term administration of Hpp11 on BP and evaluate the mechanisms underlying its antihypertensive properties in CAF-diet fed rats presenting diet-induced HTN **[Manuscript 3].**

4. To identify antihypertensive peptides from Hpp11 *(Chapter 4)*.

The identification of the bioactive peptides in the antihypertensive hydrolysate Hpp11 is important, considering their potential for HTN maintenance. Thus, chicken foot hydrolysate Hpp11 peptide profile was determined through chromatographic analyses and the potential antihypertensive effect of identified peptides was evaluated in SHR. **[Manuscript 4].**

5. **To study the bioavailability and the mechanism of action and to establish the molecular mechanisms involved in the antihypertensive effect of AVFQHNCQE *(Chapter 5)*.**

Antihypertensive peptides could reduce BP through different mechanisms than ACE inhibition. In this sense, enhancement of NO bioavailability or direct effect on vascular relaxation have been described as mechanisms involved in the antihypertensive effect of bioactive peptides. In addition, the absorption of bioactive peptides in the GIT is generally considered as requisite to exert their functionalities. Thus, the mechanisms underlying the antihypertensive effect of chicken foot-derived peptide AVFQHNCQE and its bioavailability are needed to be evaluated.

a) To establish the mechanism of action of AVFQHNCQE and to study the molecular mechanisms operating in the BP-lowering effect of this peptide in SHR. **[Manuscript 5]** and **[Manuscript 6]**
b) To study chicken foot-derived peptide AVFQHNCQE bioavailability. **[Manuscript 6]**

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EXPERIMENTAL DESIGNS

EXPERIMENTAL DESIGNS

Different experimental designs were used to assess the main hypothesis and reach the experimental objectives previously described in this thesis.

1- Obtainment of chicken foot hydrolysates with antihypertensive properties.

The experimental design used to obtain hydrolysates presenting antihypertensive properties from chicken foot proteins is described in **Figure 1**. Briefly, chicken feet were disrupted into powder, heat pre-treated and subjected to proteolytic hydrolysis under conditions (pH, time, enzyme...). Optimal hydrolysis conditions were those that allow the obtainment of hydrolysates from chicken feet showing high ACE inhibitory activity. Nevertheless, considering that high *in vitro* ACE activity does not always correspond to the same activity *in vivo*, the hydrolysates showing potent ACE inhibitory activity were administered to SHR and BP was recorded before and 2, 4 ,6 8, 24 and 48 h post-administration by the *Tail cuff* method.

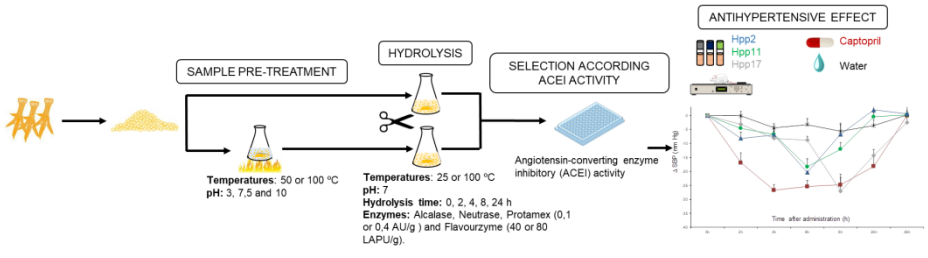

Figure 1: General procedure to obtain hydrolysates from chicken foot proteins presenting antihypertensive activity.

2- Chicken foot hydrolysate Hpp11 antihypertensive dose optimisation and study of the molecular mechanisms involved in its antihypertensive effect and the possible hypotensive effect in normotensive state.

To optimise the dose to administer of chicken foot hydrolysate Hpp11 to obtain the most effective antihypertensive effect, three different doses (85, 55 and 25 mg/kg bw) were acutely administered to SHR and BP was registered before and 2, 4, 6 8, 24 and 48 h post-administration by the *Tail cuff* method. In addition, an experiment with WKY rats was carried out to discard Hpp11 hypotensive effects in normotensive conditions. The molecular mechanisms underlying Hpp11 antihypertensive effect were studied through the determination of plasmatic ACE activity in plasma in SHR after 6 h post-Hpp11 administration. Moreover, considering that other mechanisms such as the RAAS could be involved, the effect of Hpp11 on vascular reactivity was also studied in pre-contracted aortic rings preparations from SD rats.

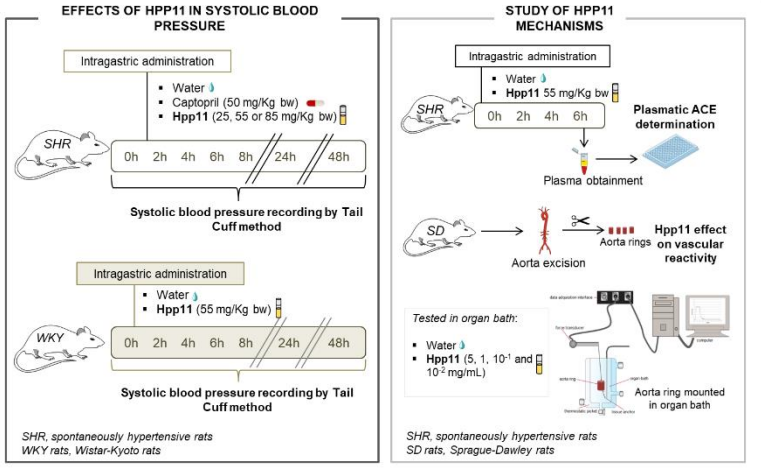

Figure 2: Experimental procedure followed to determine Hpp11 optimal dose to obtain the maximum antihypertensive effect, to discard possible Hpp11 hypotensive effect and to study the mechanisms underlying Hpp11 BP lowering effect.

3- Long-term administration effects on blood pressure of chicken foot hydrolysate Hpp11 in diet-induced hypertensive rats and the mechanisms involved in Hpp11 effect.

To evaluate the effects of chicken foot hydrolysate Hpp11 in cafeteria-diet fed rats after long-term administration and to evaluate the mechanisms involved in the long term effect of Hpp11 (**Figure 3**). Rats were fed standard or CAF diet for 12 weeks, and during the final 3 weeks, CAF diet-fed animals were administered vehicle (CAF+VH), 50 mg/kg bw Captopril (CAF+Captopril) or 55 mg/kg bw of Hpp11 (CAF+Hpp11). STD group was also administered VH (STD+VH). BP was recorded weekly. Body weight and plasmatic lipid and glucose levels were determined at the end of the experiment. Moreover, liver GSH levels, plasmatic ACE activity and the endothelial gene expression of eNOS, KLF-2, Sirt-1, NOX4 and ET-1 were studied to elucidate the mechanisms underlying the Hpp11 effect.

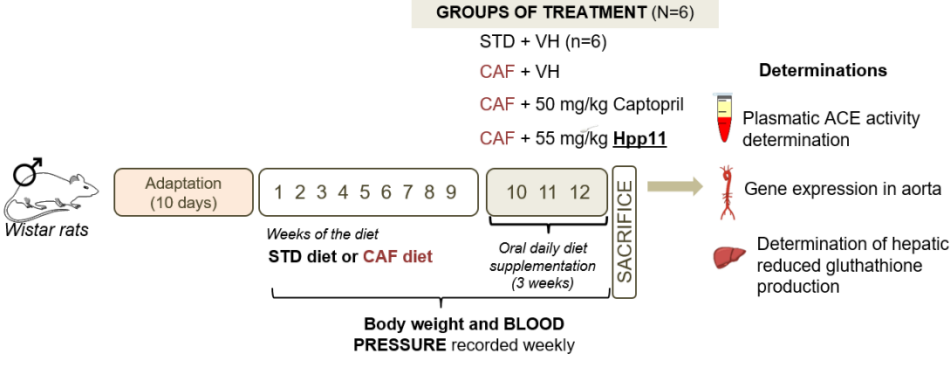

Figure 3: Experimental design to evaluate the long term administration effects of chicken foot hydrolysate Hpp11 in diet-induced rats and the mechanisms involved in its BP lowering effect.

4- Identification of antihypertensive peptides present in chicken foot hydrolysate Hpp11.

Peptide identification was performed in order to identify those responsible of the antihypertensive activity exhibited by chicken foot hydrolysate Hpp11. Therefore, Hpp11 proteins-peptides were separated in fractions according to their molecular size and hydrophobicity. Nine novel peptides were identified in the <3KDa fraction from Hpp11. Five of them showed an ACE inhibitory activity (IC_{50}) values lower than 100 μM. The antihypertensive effect of oral administration of the identified peptides was evaluated in SHR.

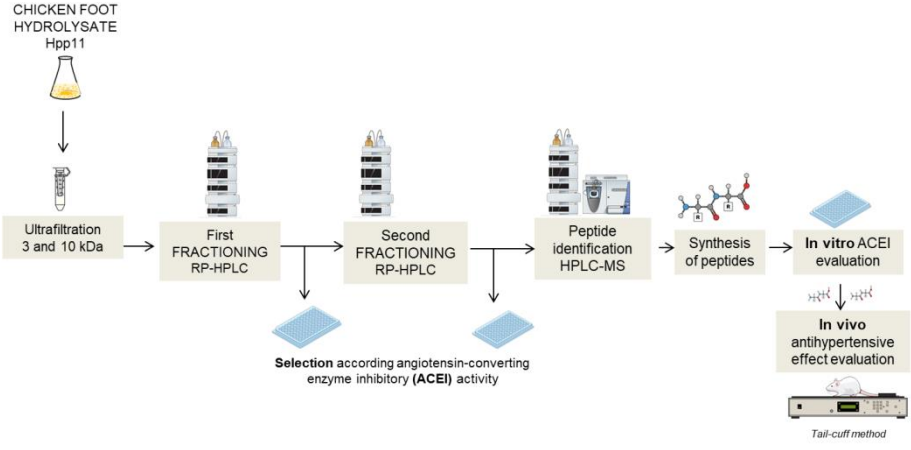

Figure 4: Experimental design to identify antihypertensive peptides from chicken foot hydrolysate.

5- Evaluation of the NO and PGI_2 implication in AVFQHNCQE antihypertensive effect. Additionally, evaluation of the molecular mechanisms underlying AVFQHNCQE antihypertensive effect.

To elucidate the implication of NO and PGI_2 in peptide antihypertensive effect, rats administered the peptide (10 mg/kg bw) or water were also treated with L-NAME (30 mg/kg bw), an inhibitor of

NO synthesis, and indomethacin (5 mg/kg bw), an inhibitor of PGI_2 synthesis. BP was recorded before and 6 h after administration (**Figure 5**). Additional experiment was performed using SHR that were administered the peptide (10 mg/kg bw) or water. After 6 h of administration rats were sacrificed and plasma, liver and aorta were extracted to perform different determinations. Hepatic GSH levels, plasmatic ACE activity and the endothelial gene expression of eNOS, KLF-2, Sirt-1, NOX4 and ET-1 were studied (**Figure 5**).

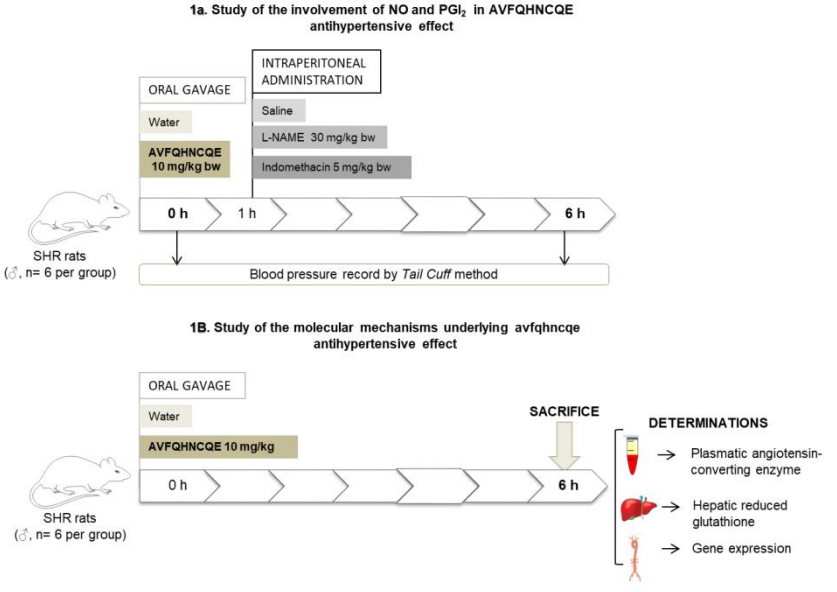

Figure 5: Experimental procedure followed to determine NO and PGI_2 implication in the AVFQHNCQE antihypertensive effect. Additional experiment was conducted in SHR to elucidate the mechanisms underlying the antihypertensive effect exhibited by AVFQHNCQE.

6- Evaluation of AVFQHNCQE bioavailability after oral administration and evaluation of its opioid-like effect.

The peptide was subjected to *in vitro* and *in silico* simulated gastrointestinal digestion. Samples from *in vitro* simulated digestion were analysed by HPLC-MS. Then, peptide was administered to Wistar

rats to study its bioavailability after oral administration. Plasma from 0 h and plasma from 30, 60 and 90 min post-administration were collected and analysed by HPLC-MS. To evaluate the peptide transport through intestinal barrier an *in vitro* with Caco-2 cells was used. The content from apical chamber and basolateral chamber from the insert were collected and analysed by HPLC-MS.

Additional experiment was conducted to evaluate the opioid-like effect of AVFQHNCQE. Thus, SHR were administered by oral gavage the peptide or water and subcutaneously injected naloxone or saline. BP was measured in these animals before and 6 h post-peptide administration.

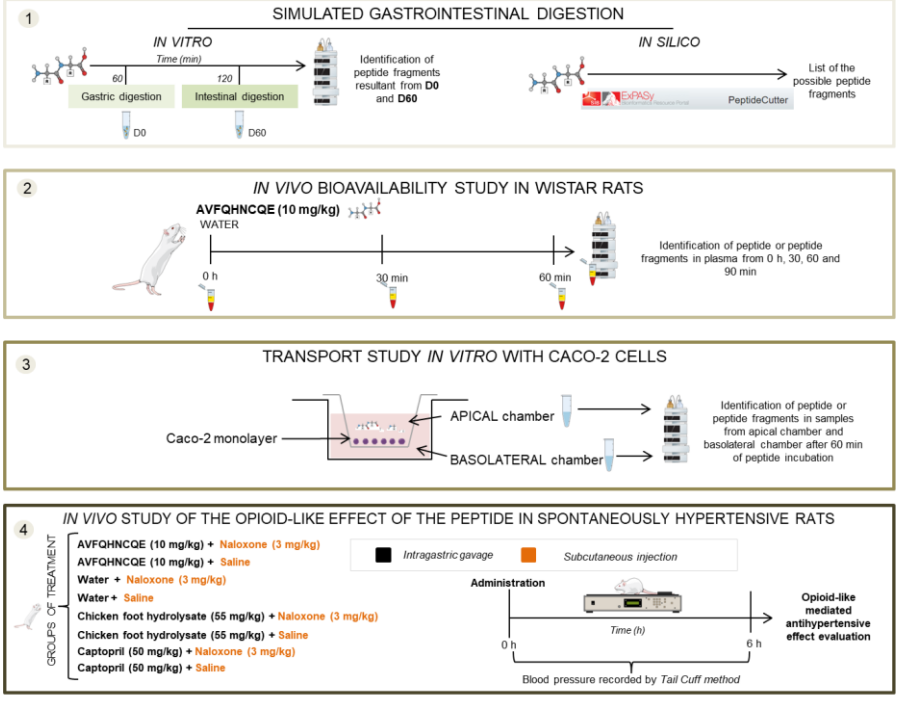

Figure 6: Experimental procedure followed to study the peptide bioavailability and its possible opioid-like effect.

RESULTS

CHAPTER 1

To obtain antihypertensive hydrolysates from chicken foot proteins

MANUSCRIPT 1

Objective

To determine the optimal hydrolysis conditions to obtain chicken foot hydrolysates presenting high ACE inhibitory activity *in vitro* but also presenting antihypertensive effect *in vivo* when administered to SHR.

Antihypertensive activity of hydrolysates from chicken foot proteins

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Keywords: Angiotensin-converting enzyme inhibition; by-products; functional foods; dietary proteins; antihypertensive peptides; hypertension.

Abbreviations: ACE, angiotensin-converting enzyme; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; SHR, spontaneously hypertensive rats; WKY, wistar kyoto rats; ACEI, angiotensin converting enzyme inhibitory; D_H, degree of hydrolysis.

Abstract

Purpose: Dietary protein hydrolysis was shown to be a successful strategy for producing antihypertensive peptides. The aim of this study was to obtain antihypertensive hydrolysates from chicken feet proteins.

Methods: In this study, we optimize the hydrolysis conditions to obtain hydrolysates from chicken feet with antihypertensive properties. The effects of hydrolysis temperature and time on angiotensin-converting enzyme inhibitory (ACEI) activity were determined. Moreover, their potential antihypertensive activity was also evaluated in spontaneously hypertensive rats (SHR).

Results: Chicken feet samples heat treatment at 100 ºC and posterior enzymatic hydrolysis at 50 ºC with Neutrase and Protamex enzymes (0.4 AU/g) were the hydrolysis conditions to produce ACEI hydrolysates. The hydrolysates obtained showed low values of IC_{50} (9.56 - 40.37 µg protein/mL). 3 hydrolysates were selected according their high ACE inhibition and demonstrated antihypertensive effect after their administration (5 mL hydrolysate/kg body weight) to SHR.

Conclusions: Chicken feet are suitable raw materials for producing antihypertensive hydrolysates that could be used to formulate antihypertensive functional foods and nutraceuticals. The results of this research open doors to the use of these hydrolysates to treat hypertension avoiding the side effects usually accompanied with the treatment with antihypertensive drugs.

1. Introduction

According to the World Health Organization, cardiovascular disease (CVD) is the leading cause of death worldwide, and it represented 31 % of all deaths in 2015 [1]. One of the most controllable risk factors associated with CVD events is hypertension, which affects nearly 45 % of the population [2].

The renin–angiotensin–aldosterone system (RAAS) plays an important role in the maintenance of arterial blood pressure (BP) and the liquid/electrolyte balance [3, 4]. One of its main components is the angiotensin-converting enzyme (ACE) [EC 3.4.15.1]. This glycosylated zinc dipeptidyl-carboxypeptidase catalyzes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II, which is the major bioactive molecule of the RAAS. In addition, ACE inactivates bradykinin, a potent vasodilator. Synthetic ACE inhibitors, like Captopril and Enalapril, are widely used for the treatment of CVD and renal disease. However, some undesirable side effects such as chronic cough, taste disturbances, skin rashes, and angioedema were described for the ACE inhibitors [5]. Therefore, the investigation of new natural product-based ACE inhibitors could greatly benefit hypertensive patients.

Dietary protein hydrolysis has been shown to be a successful strategy to produce ACE-inhibitory (ACEI) and/or antihypertensive peptides [6]. Several vegetal and animal food proteins such as legumes [7], wheat, mushrooms, bitter melon seeds, spinach [8], milk and eggs [9], meat muscle [10], fish [11] oyster or squid [12] have been shown to produce hydrolysates with high ACEI activity.

By-products generated in industrial food processing can also be used in the production of hydrolysates with ACEI activity [13]. Every year, tons of protein-rich secondary products are disposed of as waste from food manufacturing, producing environmental and economic problems [14, 15]. To reduce these problems, alternative techniques for revaluing these by-products, besides converting them into livestock feed and organic fertilizers, are being investigated [13, 15]. Chicken feet, which represent 3.5-4.0 % of the live weight [16], are a protein-rich poultry by-product that could be used as a source of inexpensive antihypertensive hydrolysates. In this sense, this by-product is especially rich in collagen (72.5 % of total protein content) [17], and this protein is a source of peptides with different bioactivities, including ACEI activity [18].

In the present study, hydrolysates obtained after an enzymatic treatment of chicken feet were selected according to their ACEI activity. Moreover, the potential beneficial effects of 3 chosen hydrolysates were assessed in an animal model of hypertension.

2. Materials and methods

2.1. Chemicals and reagents

Chicken feet from *Gallus gallus domesticus* were provided by a local farm (Granja Gaià, La Riera de Gaià, Spain). Neutrase® 0.8 L (EC 3.4.24.28, 0.8 AU-N/g from *Bacillus amyloliquefaciens*) and Protamex® (EC 3.4.21.62 and 3.4.24.28, 1.5 AU/g from *Bacillus licheniformis and Bacillus amyloliquefaciens*) were kindly provided by from Novozymes (Lyngby, Denmark). ACE (peptidyl-dipeptidase A, EC 3.4.15.1) and picrylsulfonic acid solution (5 %, w/v) were purchased from Sigma Chemical (St. Louis, MO, USA), and *o*-aminobenzoylglicil-*p*-

nitrofenilalanilprolina (o-Abz-Gly-p-Phe(NO_2)-Pro-OH) was provided by Bachem Feinchemikalien (Bubendorf, Switzerland). All other chemicals were obtained from common commercial sources and were analytical grade.

2.2. Preparation of chicken feet hydrolysates

Chicken feet were cleaned, crushed, lyophilized and milled using a Fagor BV-850 beater (Fagor Electrodomésticos S. Coop., Mondragón, Spain). The obtained powder was sieved using a 2 mm pore size sieve to obtain fine chicken feet powder. Samples were prepared dissolving chicken feet powder in Milli-Q water at 20 mg/mL and vortexing during 1 min. Posteriorly, a heating treatment was carried out at 100 ºC and pH 3.0, 7.5, or 10.0 with agitation over 1.5 h. After that, the pH of all samples was adjusted to 7.0 with HCl or NaOH 1 M, and the hydrolysis enzyme was added at a final concentration of 3.97 µL/mL for Neutrase and 2.67 µg/mL for Protamex. The used enzyme/substrate ratio for all enzymes was 0.4 AU/g protein. Hydrolysis was conducted at pH 7.0 and 250 rpm in a MaxQ Orbital Shaker Thermo Scientific (Thermo Fisher Scientific, USA) at a controlled temperature of 25 or 50 ºC for 0, 2, 4 or 24 h. At least duplicate hydrolysis was carried out in all conditions of hydrolysis. Inactivation of enzymes was achieved by heating at 80 ºC for 10 min. The hydrolysates were centrifuged at 10,000 xg for 20 min at 4 ºC. The supernatants obtained were filtered using 0.45 µm filters and kept at -20 ºC until used. In addition, samples without chicken feet heating treatment and hydrolysis enzymes were also prepared. These samples were obtained from chicken feet powder dissolved in Milli-Q water (20 mg/mL) by vortexing during 1 min, then centrifuged at 10,000 xg for 20 min at 4 ºC, and filtered using 0.45 µm filters.

2.3 Characterization of chicken feet powder and hydrolysates

Moisture, ash, fat and protein content of chicken feet or hydrolysates was determined according to the AOAC methods [19]. Protein content was determined by Kjeldahl method, multiplying the determined nitrogen content by 6.25. The degree of hydrolysis (D_H) was assayed by measuring the increase in free amino groups using the trinitro-benzene-sulfonic acid (TNBS) method according to Adler-Nissen (1979) [20]. The total number of amino groups was determined in a sample that was 100 % hydrolyzed at 110 °C for 24 h in 6 M HCl. All determinations were carried out in at least duplicate. The data shown in Table 2, concerning the protein content and D_H, are mean values of at least two different hydrolysates in the same conditions. All data are expressed as the mean ± standard deviation (SD).

2.4 Determination of ACE-inhibitory activity

ACEI activity was measured by fluorescence following the method of Sentandreu and Toldrá (2006) with some modifications according to Guerrero et al. [22]. ACE working solution was diluted with 0.15 M Tris buffer (pH 8.3) containing 0.1 µM of $ZnCl_2$ with 0.04 U/mL of enzyme in the final reaction solution. A total of 40 µL of sample, distilled water or this enzyme working solution were added to each microtiter-plate well and adjusted to 80 µL by adding distilled water if necessary. The enzymatic reaction started by adding 160 µL of 0.45 mM o-Abz-Gly-p-Phe(NO_2)-Pro-OH dissolved in 150 mM Tris-base buffer (pH 8.3), containing 1.125 M NaCl, and the mixture was incubated at 37 °C. The sample fluorescence was recorded at 30 min using a multi-scan microplate fluorimeter (FLUOstar optima, BMG Labtech, Offeuburg, Germany). Excitation and emission wavelengths were 360 and 400 nm, respectively. The software used to process the data were FLUOstar control (version 1.32 R2, BMG Labtech).

ACEI activity of all hydrolysates were determined as a percentage of inhibition of hydrolysates diluted in Milli Q water (1:1, v:v). Hydrolysates with ACEI activity ≥ 90 % were selected, and IC_{50} was determined. IC_{50} is the concentration of each hydrolysate (µL of hydrolysate or µg of protein/mL of hydrolysate), which reduces enzyme activity by half.

The determination of ACEI activity of samples was performed in at least duplicate. The data shown are mean values of ACEI activity of at least two different hydrolysates in the same conditions.

2.5 Measurement of arterial blood pressure

Male SHR rats (17–20-week-old, weighing 300–350 g) were obtained from Charles River Laboratories España S.A. (Barcelona, Spain). Animals were singly housed in animal quarters at 22 °C with light/dark period of 12 h. They were fed with the standard diet based on chow Panlab A04 (Panlab, Barcelona, Spain) and tap water *ad libitum*.

Antihypertensive activity of selected hydrolysates was determined by the tail-cuff method [23]. The original method for measuring arterial blood pressure using the tail cuff provides only systolic blood pressure (SBP) values, but the equipment used in this study, LE 5001 (Letica, Hospitalet, Barcelona, Spain), has a high-sensitivity pulse transducer coupled with an accurate microprocessor program and allows for us to distinguish between SBP and diastolic blood pressure (DBP). Before starting the experiment, all animals were accustomed to the process through a 3 weeks training period.

The different hydrolysates were acutely administered to the rats by gastric intubation between 9 and 10 am. The administered dose of the hydrolysates was 5 mL/kg body weight (bw). Negative and positive control groups received 1.5 mL of water or Captopril (50 mg/kg bw, dissolved in water), respectively. SBP and DBP were measured in the

animals before the treatment administration and 2, 4, 6, 8, 24 and 48 h post-administration. Changes in SBP and DBP were expressed as the differences between the mean values of these variables before and after the administration. Data are expressed as the mean values ± standard error of the mean (SEM) for a minimum of six experiments.

The animal protocol followed in this study was approved by the Bioethical Committee of Universitat Rovira i Virgili (European Comission Directive 86/609) and the Spanish Royal Decree 223/1988.

2.6 Statistical Analysis

To assess the effect of chicken feet protein hydrolysis conditions on ACEI activity, data were analyzed by Student's T-test. Data obtained in the determination of antihypertensive activity were analyzed using two-way ANOVA (Duncan's test). All analyses were carried out using IBM SPSS statistics 20.0 software (SPSS, Inc, Chicago, IL, USA). Differences between the means were significant when $p < 0.05$.

3. Results and discussion

Protein-rich by-products from non-mammalian species, such as poultry or fish, have received considerable attention in recent years [24]. In this study, chicken feet were used as a protein source to obtain hydrolysates with ACEI properties. Although other chicken parts have been utilized as source of hydrolysates with ACEI activity [25–27] chicken feet have never been used as far as we know.

Protein, fat and moisture content of the used chicken feet were 20.1, 15.03 and 60.5 %, respectively. These contents were quite similar to those previously described for this poultry by-product (17.42, 12.04 and 62.05 %, respectively) [28]. Processing of raw feet to powder resulted in a homogeneous material composed of small particles that was more

accessibility to the proteases and enriched in protein (44.3 vs 20.1 %). Similar protein content values were reported for chicken essence residues used as raw material by Chen et al. [29] to obtain antihypertensive hydrolysates. ACEI activity of the supernatant obtained by centrifugation of chicken feet powder dissolved in water was 28.62 %, similar to those reported previously by chicken bone or leg protein solutions [27, 30].

The accessibility of proteases to proteins and therefore peptide releasing can be modulated by many factors like their solubilization and native or denatured state, which depends on environmental factors (i.e., pH, temperature). In this regard, in this study, a heating treatment at 100 °C before hydrolysis was applied to the chicken feet powder dissolved in water. Heat pretreatment of chicken breast and thigh in water in the obtaining of hydrolysates with ACEI activity has been previously used [31]. In addition, since protein solubilization is depending on pH, the samples were subjected to different pH prior hydrolysis treatment.

The effect of hydrolysis temperature of 25 and 50 °C on ACEI activity was studied. Hydrolysate ACEI activity, expressed as a percentage did not show significant differences associated with hydrolysis temperatures. However, IC_{50}, expressed as volume (µL), was lower in the hydrolysates prepared at 50 °C than at 25 °C (Table 1). It is known that the lower IC_{50} value, the higher the ACE inhibitory activity. Therefore, 50 °C was chosen as optimal temperature to perform the chicken feet hydrolysis. In addition, hydrolysis times shorter than 24 h (2 and 4 h) were studied. Protein content, D_H and ACEI activity of the hydrolysates were determined (Table 2). It was observed that only the samples at 0 h (without hydrolysis) showed an ACEI activity of less than 90 %. These results demonstrated that the use of these hydrolysis

conditions (time, temperature) is a great strategy to obtain hydrolysates presenting high ACEI activity. In addition, the IC_{50} value was determined for each hydrolysate and expressed as the volume and protein concentration. The expression of this value in volume indicates the necessary hydrolysate volume to inhibit the enzyme by 50 % under the assay conditions. Therefore, this value is indicative of the pharmacological potency, given that the lower the IC_{50}, expressed in volume, the higher the potency of ACEI of the hydrolysate assayed [32]. All hydrolysates showed a strong ACEI activity, although differences in the IC_{50} values between the different hydrolysates can be noted (Table 2). ACEI activities of hydrolysates ranged among 0.5 ± 0.0 and 1.4 ± 0.0 µL. These inhibitory potencies are comparable to others obtained from milk fermented with *Enterococcus faecalis* [33]. Moreover, IC_{50} was also expressed in protein concentration (µg/mL). It is an indicator of the pharmacological specificity or the specificity of the obtained peptide mixture against ACE, regardless of the protein concentration of the fraction [33]. ACEI activities ranged among 9.6 ± 3.1 and 40.4 ± 0.5 µg/mL (Figure 1). These results clearly reveal important ACEI potency in chicken foot hydrolysates. Chicken legs have been previously reported as a source of ACEI hydrolysates, but the hydrolysates obtained showed higher IC_{50} than ones obtained in this study from chicken feet. In fact, Indonesian native boneless chicken leg proteins hydrolyzed with different *Bacillus cereus* enzymes and broiler chicken leg bones hydrolyzed with Alcalse showed IC_{50} ranging from 945 to 2580 µg/mL [34, 35]. In addition, hydrolysates with ACEI activity from other chicken by-products have also been obtained. In this sense, hydrolysates derived from spent hen meat proteins treated with Pepsin or Pepsin-Pancreatin showed IC_{50} values of 646 and 424 µg/mL, respectively [36], and hydrolysates obtained

from skin proteins of thigh or breast treated with Alcalase or Pepsin-Pancreatin, respectively, showed IC_{50} values of 550 and 640 μg/mL, respectively [37]. Moreover, chicken blood proteins treated with Alcalase showed ACEI properties, but the IC_{50} value reported for this by-product (340 μg/mL) was higher than the obtained in this study.

However, *in vitro* ACEI activity is not always accompanied with *in vivo* antihypertensive effect [38]. In fact, some bioactive peptides can be hydrolyzed and transformed into inactive peptides during gastrointestinal digestion [39]. In this study, the antihypertensive effect of 3 different chicken foot hydrolysates (Hpp8, Hpp11 and Hpp23) was tested in SHR, the experimental animal model that best mimics essential hypertension in humans [40]. Figure 2 shows the *in vitro* ACEI activity of these hydrolysates expressed in percentage versus the volume of hydrolysate.

Prior to the administration of the different hydrolysates, SHR presented SBP and DBP values of 202 ± 7.5 and 174 ± 9.3 mmHg, respectively. Neither SBP nor DBP of SHR administered water changed significantly during the 48 h of experiment duration (Figure 3). As expected, the administration of Captopril produced an important decrease in SBP and DBP of SHR reaching the maximum decrease at 4 and 6 h post-administration. These variables returned to baseline 48 h after Captopril administration. In addition, oral administration of the selected chicken feet hydrolysates caused a significant decrease in both SBP and DBP compared to water group ($p \leq 0.05$). These results indicate that the ACEI peptides present in the chicken feet hydrolysates, or shorter forms generated by gastrointestinal digestion, can be efficiently absorbed through the intestine of the animals in an active form. In this sense, the maximum SBP decreases caused by the administration of the hydrolysates (-20.3 ± 0.5, -18.4 ± 2.8, and -27.1 ±

4.8 mmHg for Hpp8, Hpp11 and Hpp23, respectively) to SHR, were similar to the decrease produced after the administration of Captopril (-26.7 ± 2.1 mmHg) ($p > 0.05$) (Figure 3A). Similar maximum SBP decreases were reported in a previous study carried out with a comparable dose of a hydrolysate obtained from chicken leg bone with Alcalase [30]. In addition, a comparable decrease in SBP was reported after oral administration of spent hen meat proteins hydrolyzed with Pepsin and Pepsin-Pancreatin, but a 4-fold higher dose was administered in this study [36]. Regarding DBP, rats given the tested hydrolysates reached similar values compared to those reported for normotensive animals [41].

Curiously, the tested hydrolysates presented different decreasing patterns, while Hpp8 and Hpp11 presented the maximum decrease in SBP 6 h post-administration, and Hpp23 showed its maximum antihypertensive effect at 8 h post-administration. Moreover, the antihypertensive effect of Hpp23 was more prolonged over time, remaining at least 24 h post-administration. In addition, the antihypertensive effect of Hpp8 and Hpp11 on DBP was again very similar, presenting both hydrolysates a main decrease 6 h post-administration. However, the maximum decrease in DBP caused by the Hpp23 was 2 h before, at 4 h post-administration. Values of DBP returned to initial values, 24 or 48 h post-administration of Hpp8 and Hpp11 or Hpp23, respectively. Therefore, these findings indicate that the peptides responsible of antihypertensive effect could be different in the different hydrolysates since the antihypertensive effect pattern is not the same for all them. In fact, Hpp8 and Hpp11 peptides exert quicker antihypertensive effects than Hpp23 peptides, and they are rapidly metabolized into inactive products leading to the subsequent reduction in activity.

4. Conclusions

This study shows that chicken feet are suitable raw materials for producing antihypertensive hydrolysates that could be used to formulate antihypertensive functional foods and nutraceuticals. Moreover, their use will enhance the revalorization of these waste products and make slaughterhouses more environmentally friendly. Further studies to identify the peptides responsible for these activities are in progress.

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Conflict of interest

The authors declare no conflict of interest.

References

1. WHO (2017) Cardiovascular diseases
2. Girgih AT, Udenigwe CC, Li H, et al (2011) Kinetics of enzyme inhibition and antihypertensive effects of hemp seed (Cannabis sativa L.) protein hydrolysates. JAOCS, J Am Oil Chem Soc 88:1767–1774
3. Skeggs LT, Dorer FE, Kahn JR, et al (1976) The biochemistry of the renin-angiotensin system and its role in hypertension. Am J Med 60:737–748 . doi: 10.1016/0002-9343(76)90888-3
4. Atlas SA, Case DB, Sealey JE, et al (1979) Interruption of the renin-angiotensin system in hypertensive patients by captopril induces sustained reduction in aldosterone secretion, potassium retention and natruiresis. Hypertension 1:274–280
5. Antonios TF, MacGregor GA (1995) Angiotensin converting enzyme inhibitors in hypertension: potential problems. J Hypertens Suppl 13:S11–S16
6. Martínez-Maqueda D, Miralles B, Recio I, Hernández-Ledesma B (2012) Antihypertensive peptides from food proteins: a review. Food Funct 3:350
7. Boschin G, Scigliuolo GM, Resta D, Arnoldi A (2014) ACE-inhibitory activity of enzymatic protein hydrolysates from lupin and other legumes. Food Chem 145:34–40
8. Daskaya-Dikmen C, Yucetepe A, Karbancioglu-Guler F, et al (2017) Angiotensin-I-converting enzyme (ACE)-inhibitory peptides from plants. Nutrients 9:316–334
9. Aleixandre A, Miguel M, Muguerza B (2008) Péptidos antihipertensivos derivados de proteínas de leche y huevo. Nutr Hosp 23:313–318
10. Ahhmed AM, Muguruma M (2010) A review of meat protein hydrolysates and hypertension. Meat Sci 86:110–118
11. Huang J, Liu Q, Xue B, et al (2016) Angiotensin-I-converting enzyme inhibitory activities and in vivo antihypertensive effects of sardine protein hydrolysate. J Food Sci 81:H2831–H2840
12. Udenigwe CC, Adebiyi AP, Doyen A, et al (2012) Low molecular weight flaxseed protein-derived arginine-containing peptides reduced blood pressure of spontaneously hypertensive rats faster than amino acid form of arginine and native flaxseed protein. Food Chem 132:468–475
13. Lasekan A, Abu Bakar F, Hashim D (2013) Potential of chicken by-products as sources of useful biological resources. Waste Manag 33:552–565
14. Lafarga T, Hayes M (2014) Bioactive peptides from meat muscle and by-products: Generation, functionality and application as functional ingredients. Meat Sci 98:227–239
15. Valta K, Damala P, Orli E, et al (2015) Valorisation opportunities related to wastewater and animal by-products exploitation by the greek slaughtering industry: current status and future potentials. Waste and biomass valorization 6:927–945
16. Sams AR (2001) Poultry meat processing. 264.
17. Braz Í, Kênia T, Bezerra A, et al (2018) Optimal conditions for obtaining collagen from chicken feet and its characterization. 2061:1–7
18. Alemán A, Pérez-Santín E, Bordenave-Juchereau S, et al (2011) Squid gelatin hydrolysates with antihypertensive, anticancer and antioxidant activity. Food Res Int 44:1044–1051
19. AOAC (1995) Official methods of analysis, 16th ed. Arlington
20. Adler-Nissen J (1979) Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. J Agric Food Chem 27:1256–1262
21. Sentandreu MÁ, Toldrá F (2006) A fluorescence-based protocol for quantifying angiotensin-converting enzyme activity. Nat Protoc 1:2423–2437

22. Guerrero L, Castillo J, Quiñones M, et al (2012) Inhibition of angiotensin-converting enzyme activity by flavonoids: Structure-activity relationship studies. PLoS One 7:1–11
23. Buñag RD (1973) Validation in awake rats of a tail-cuff method for measuring systolic pressure. J Appl Physiol 34:279–282
24. Margalef M, Bravo FI, Arola-Arnal A, Muguerza B (2017) Natural angiotensin converting enzyme (ACE) inhibitors with antihypetensive properties. In: Andrade P., Valentao P, Pereira DM (eds) Natural products targeting clinically relevant enzymes, First. Wiley-VCH Verlag GmbH & Co. , Weinheim, pp 45–67
25. Cheng FY, Wan TC, Liu YT, et al (2009) Determination of angiotensin-I converting enzyme inhibitory peptides in chicken leg bone protein hydrolysate with alcalase. Anim Sci J 80:91–97
26. Saiga A, Okumura T, Makihara T, et al (2003) Angiotensin I-converting enzyme inhibitory peptides in a hydrolyzed chicken breast muscle extract. J Agric Food Chem 51:1741–1745
27. Saiga A, Iwai K, Hayakawa T, et al (2008) Angiotensin I-converting enzyme-inhibitory peptides obtained from chicken collagen hydrolysate. J Agric Food Chem 56:9586–9591
28. Liu D., Lin Y., Chen M. (2001) Optimum condition of extracting collagen from chicken feet and its characetristicscs. Asian-australasian J Anim Sci 14:1638–1644
29. Chen Y-H, Liu Y-H, Yang Y-H, et al (2002) Antihypertensive effect of an enzymatic hydrolysate of chicken essence residues. Food Sci Technol Res 8:144–147
30. Cheng FY, Wan TC, Liu YT, et al (2008) A study of in vivo antihypertensive properties of enzymatic hydrolysate from chicken leg bone protein. Anim Sci J 79:614–619
31. Sangsawad P, Roytrakul S, Choowongkomon K, et al (2018) Transepithelial transport across Caco-2 cell monolayers of angiotensin converting enzyme (ACE) inhibitory peptides derived from simulated in vitro gastrointestinal digestion of cooked chicken muscles. Food Chem 251:77–85
32. Fuglsang A, Nilsson D, Nyborg NCB (2002) Cardiovascular effects of fermented milk containing angiotensin- converting enzyme inhibitors evaluated in permanently catheterized , spontaneously hypertensive rats. Society 68:3566–3569 .
33. Muguerza B, Ramos M, Sánchez E, et al (2006) Antihypertensive activity of milk fermented by Enterococcus faecalis strains isolated from raw milk. Int Dairy J 16:61–69
34. Yuliatmo R, Fitriyanto N. A, Bachruddin Z S, Jamhari, Y. E (2017) Increasing of angiotensin converting enzyme inhibitory derived from chicken leg. Int Food Res J 24:1799–1804
35. Cheng F-Y, Liu Y-T, Wan T-C, et al (2008) The development of angiotensin I-converting enzyme inhibitor derived from chicken bone protein. Anim Sci J 79:122–128
36. Udenigwe CC, Girgih AT, Mohan A, et al (2017) Antihypertensive and bovine plasma oxidation-inhibitory activities of spent hen meat protein hydrolysates. J Food Biochem 41:1–8 . doi: 10.1111/jfbc.12378
37. Onuh JO, Girgih AT, Malomo SA, et al (2015) Kinetics of in vitro renin and angiotensin converting enzyme inhibition by chicken skin protein hydrolysates and their blood pressure lowering effects in spontaneously hypertensive rats. J Funct Foods 14:133–143
38. FitzGerald RJ, Meisel H (2000) Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. Br J Nutr 84 Suppl 1:S33-7
39. Jao C-L, Huang S-L, Hsu K-C (2012) Angiotensin I-converting enzyme

inhibitory peptides: Inhibition mode, bioavailability, and antihypertensive effects. BioMedicine 2:130–136

40. FitGerald R., Murray B., Walsh DJ (2004) Hypotensive peptides from milk proteins. J Nutr 134:980S–988S
41. Girgih AT, Nwachukwu ID, Onuh JO, et al (2016) Antihypertensive properties of a pea protein hydrolysate during short- and long-term oral administration to spontaneously hypertensive rats. J Food Sci 81:H1281–H1287

TABLES

Table 1. ACEI activity represented as percentage or IC_{50} (μL) of hydrolysates obtained from chicken feet powder solutions with Neutrase or Protamex at 25 or 50 °C during 24 h.

Heat treatment conditions[a]	Hydrolysis conditions					
			Temperature			
			25 °C		50 °C	
pH	Enzyme	Time (h)	ACEI activity[b] (%)	IC_{50} (μL)	ACEI activity[b] (%)	IC_{50} (μL)
3.0	Neutrase	24	87.9 ± 3.0	1.5 ± 0.7	91.0 ± 1.8	0.9 ± 0.0
7.5			91.5 ± 1.2	1.4 ± 0.7	93.0 ± 0.5	0.8 ± 0.3
10.0			91.5 ± 7.9	1.7 ± 1.4	93.1 ± 3.9	1.2 ± 0.1
3.0	Protamex	24	92.3 ± 1.4	1.1 ± 0.2	93.1 ± 0.6	0.5 ± 0.0**
7.5			90.1 ± 5.8	1.1 ± 0.3	95.7 ± 3.5	0.5 ± 0.0**
10.0			96.4 ± 3.0	1.0 ± 0.6	97.4 ± 1.4	0.5 ± 0.1**

[a] Chicken feet powder solutions were treated at 100 °C prior hydrolysis at different pH conditions.

[b] Angiotensin-converting enzyme-inhibitory activity of 50 % diluted hydrolysate.

The data shown are mean values ± SD of ACEI activity of at least two different hydrolysates elaborated in the same conditions.

** Differences between the IC_{50} (μL) of the hydrolysates elaborated under different hydrolysis temperature (25 or 50 °C) (Student's t test. $p < 0.01$).

Table 2. Degree of hydrolysis (D_H), protein content and ACEI activity of hydrolysates obtained from chicken feet powder solutions treated at 100 °C and pH 3.0, 7.5 or 10.0 during 1.5 h and subsequent hydrolysis with Neutrase or Protamex at 50 °C, pH 7.0.

Samples	pH[a]	Hydrolysis conditions		D_H (%)	Protein[b] (%)	ACEI activity	
		Enzyme	Time (h)			Inhibition[c] (%)	IC_{50}[d] (µL)
Hpp1[e]	3.0	Neutrase	0	10.1 ± 0.4	0.4 ± 0.0	83.0 ± 2.5	2.7 ± 0.1
Hpp2[e]	7.5			9.7 ± 0.2	0.3 ± 0.0	71.0 ± 3.5	3.4 ± 0.1
Hpp3[e]	10.0			10.9 ± 0.2	0.3 ± 0.0	80.8 ± 1.1	2.0 ± 0.3
Hpp4[e]	3.0	Protamex	0	13.8 ± 0.1	0.5 ± 0.0	81.6 ± 5.5	4.1 ± 0.4
Hpp5[e]	7.5			14.9 ± 0.1	0.4 ± 0.0	82.6 ± 1.6	2.6 ± 0.4
Hpp6[e]	10.0			14.9 ± 0.2	0.4 ± 0.0	85.2 ± 2.8	3.1 ± 0.1
Hpp7	3.0	Neutrase	2	17.7 ± 1.6	0.6 ± 0.0	93.8 ± 1.6	0.9 ± 0.1
Hpp8	7.5			20.0 ± 1.7	0.6 ± 0.0	93.8 ± 1.0	**0.9 ± 0.1**
Hpp9	10.0			21.4 ± 5.8	0.6 ± 0.0	94.0 ± 1.5	1.0 ± 0.0
Hpp10	3.0	Protamex	2	23.1 ± 2.2	0.7 ± 0.0	94.8 ± 0.8	0.8 ± 0.2
Hpp11	7.5			20.8 ± 1.5	0.7 ± 0.0	93.9 ± 1.7	**0.6 ± 0.0**
Hpp12	10.0			22.0 ± 0.4	0.7 ± 0.0	94.8 ± 3.5	0.7 ± 0.2
Hpp13	3.0	Neutrase	4	20.5 ± 2.0	0.6 ± 0.0	101.6 ± 2.5	1.0 ± 0.0
Hpp14	7.5			23.4 ± 0.9	0.6 ± 0.0	91.4 ± 2.0	1.3 ± 0.1
Hpp15	10.0			27.1 ± 2.2	0.7 ± 0.0	91.8 ± 2.2	1.4 ± 0.0
Hpp16	3.0	Protamex	4	25.0 ± 2.4	0.7 ± 0.0	94.6 ± 0.1	0.7 ± 0.1
Hpp17	7.5			24.5 ± 1.6	0.7 ± 0.0	95.8 ± 2.7	0.6 ± 0.0
Hpp18	10.0			24.1 ± 0.2	0.7 ± 0.0	96.5 ± 4.9	0.6 ± 0.0
Hpp19	3.0	Neutrase	24	20.4 ± 0.4	0.7 ± 0.0	91.0 ± 1.8	1.0 ± 0.0
Hpp20	7.5			22.9 ± 0.1	0.7 ± 0.1	93.0 ± 0.5	0.8 ± 0.3
Hpp21	10.0			35.3 ± 3.9	0.6 ± 0.0	93.1 ± 3.9	1.3 ± 0.1
Hpp22	3.0	Protamex	24	33.7 ± 0.8	0.7 ± 0.0	93.1 ± 0.7	0.5 ± 0.0

Hpp23	7.5			28.5 ± 0.2	0.7 ± 0.0	95.8 ± 3.5	**0.5 ± 0.0**
Hpp24	10.0			28.8 ± 0.7	0.7 ± 0.0	97.4 ± 1.4	0.5 ± 0.1

[a] pH used in the heat treatment (100 ºC) of chicken feet powder dissolved in water prior to hydrolysis.

[b] Protein was measured by Kjeldahl method.

[c] Angiotensin converting enzyme inhibitory activity of 50 % diluted hydrolysate.

[d] Volume of hydrolysate needed to give the 50 % of inhibition.

[e] Chicken feet powder solution treated at 100 ºC and different pH and posteriorly added the corresponding enzyme but without hydrolysis.

FIGURES

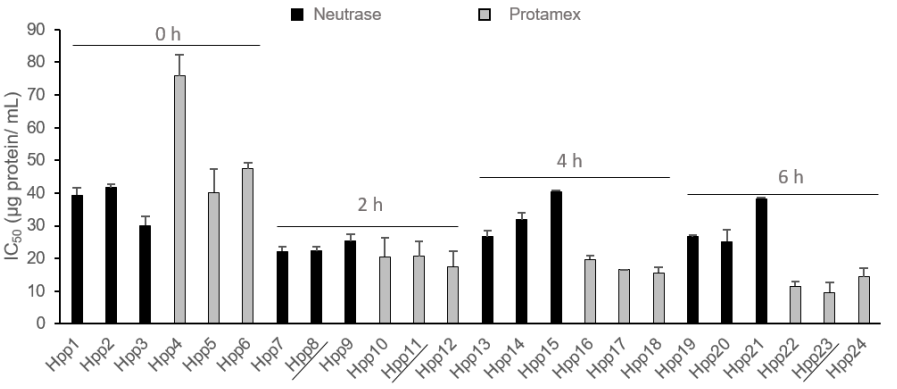

Figure 1. Graphical representation of the IC_{50}, concentration of protein (µg/mL of hydrolysate) required to inhibit 50 % angiotensin-converting enzyme activity, of the 24 hydrolysates obtained in this study. Values are the average of two replicates ± SD. Nomenclature of the hydrolysates is as described in Table 2.

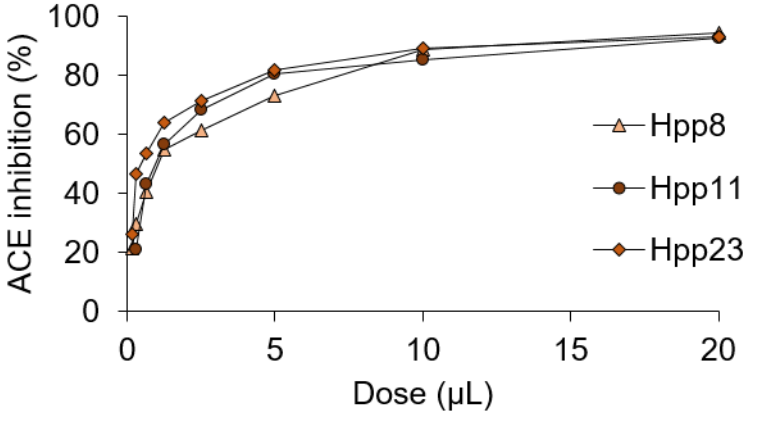

Figure 2. Dose–response curves (effect as a function of the dose in microliters) for the three selected hydrolysates. Values are the average of two replicates. Nomenclature of the hydrolysates is as described in Table 2.

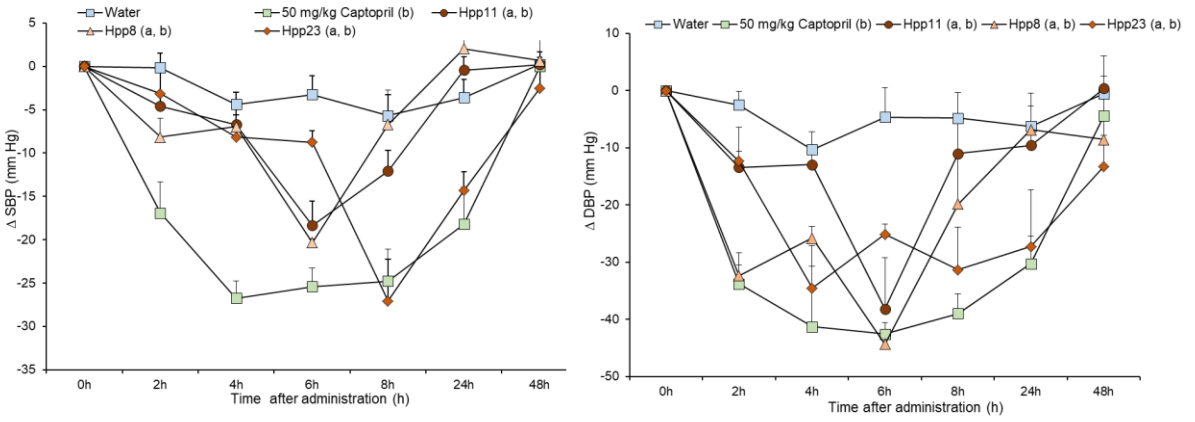

Figure 3. Decrease in systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) caused in spontaneously hypertensive rats by the administration of water, Captopril (50 mg/kg bw), and the 3 selected hydrolysates obtained from chicken feet proteins (5 mL/kg bw): Hpp8, Hpp11 and Hpp23. The data represent mean values ± SEM for a minimum of 6 rats. Different letters represent significant differences ($p < 0.01$) between hydrolysate administered group and water (a) or differences between hydrolysate administered group and captopril group (b) (Two-way ANOVA, Duncan post hoc test).

CHAPTER 2

To stablish the most effective acute dose and the mechanisms involved in Hpp11 antihypertensive effect

MANUSCRIPT 2

Objective

To determinate the most effective acute dose of a chicken foot hydrolysate Hpp11 to reduce BP in SHR, to discard a possible hypotensive effect in normotensive animals WKY rats and to establish the mechanisms underlying the antihypertensive effect of Hpp11 in SHR.

Dose-related antihypertensive properties and the corresponding mechanisms of a chicken foot hydrolysate in hypertensive rats

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Dose-Related Antihypertensive Properties and the Corresponding Mechanisms of a Chicken Foot Hydrolysate in Hypertensive Rats

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Abstract: The antihypertensive properties of different doses of a chicken foot hydrolysate, Hpp11 and the mechanisms involved in this effect were investigated. Spontaneously hypertensive rats (SHR) were administered water, Captopril (50 mg/kg) or Hpp11 at different doses (25, 55 and 85 mg/kg), and the systolic blood pressure (SBP) was recorded. The SBP of normotensive Wistar-Kyoto (WKY) rats administered water or Hpp11 was also recorded. Additionally, plasmatic angiotensin-converting enzyme (ACE) activity was determined in the SHR administered Hpp11. Moreover, the relaxation caused by Hpp11 in isolated aortic rings from Sprague-Dawley rats was evaluated. Hpp11 exhibited antihypertensive activity at doses of 55 and 85 mg/kg, with maximum activity 6 h post-administration. At this time, no differences were found between these doses and Captopril. Initial SBP values of 55 and 85 mg/kg were recovered 24 or 8 h post-administration, respectively, 55 mg/kg being the most effective dose. At this dose, a reduction in the plasmatic ACE activity in the SHR was found. However, Hpp11 did not relax the aortic ring preparations. Therefore, ACE inhibition could be the mechanism underlying Hpp11 antihypertensive effect. Remarkably, Hpp11 did not modify SBP in WKY rats, showing that the decreased SBP effect is specific to the hypertensive state.

Keywords: hypertension; protein hydrolysate; angiotensin-converting enzyme; ACE-inhibitory activity; endothelial dysfunction; bioactive peptides

1. Introduction

Cardiovascular diseases (CVD) are the leading cause of mortality in Europe, and hypertension (HTN) is one of the main CVD risk factors [1]. In this sense, the lowering of blood pressure (BP) through behavioural and pharmacological interventions has been showed to remarkably improve CVD [2]. Currently, one of the most popular pharmacologic therapies to treat HTN is based on the use of angiotensin-converting enzyme (ACE) inhibitors such as Captopril or Enalapril [3]. ACE is the key enzyme of the renin-angiotensin-aldosterone system (RAAS), which is one of the most important systems in the regulation of blood volume and systemic vascular resistance. ACE hydrolyses the decapeptide angiotensin I (Ang I) to the octapeptide angiotensin II (Ang II), which is a potent vasoconstrictor but also breaks down bradykinin, a vasodilator [4]. Although pharmacological ACE inhibitors are widely used to decrease BP, some undesirable side effects have been described for these drugs, such as angioedema, dry cough, disturbance in taste, and skin reactions, among others [5].

In consequence, the study of natural bioactive compounds has received great attention, and their use has been considered a good strategy to decrease the risk of HTN [6].

Specifically, the hydrolysis of food proteins is considered a potential source of peptides with ACE inhibitory activities and/or antihypertensive effects [7–10]. Different protein sources have been reported to release ACE inhibitor peptides, such as milk and egg [11], fish [12,13] or meat [14], among others. Considering this, the interest in proteins derived from food by-products as sources of ACE inhibitor peptides has increased. The use of food by-products allows the reuse of waste materials, making the food and agricultural industries more environmentally friendly [15–17]. In this regard, our group has used chicken feet, a poultry industry by-product, to obtain hydrolysates that present ACE inhibitory (ACEI) activity [18]. Chicken feet is considered a by-product in Spain and in most countries in Europe, and since a few years ago, the conversion of animal by-products to feed and certain legislations in some countries disallows indiscriminate dumping or landfilling of animal wastes [19]. Thus, this chicken by-product represents environmental and economic problems for meat processors if they are not correctly treated. Considering this, the chicken industry considered that it is no longer practical to discard by-products and wastes, especially when a significant amount of valuable raw materials have a strong economic potential like the production of new products and functional ingredients with a significant added-value [20]. It is known that chicken proteins, especially chicken collagen, has been demonstrated to be a source of bioactive peptides, able to inhibit ACE and exhibit antihypertensive activity [21–23]. Nevertheless, the in vitro ACEI activity and in vivo antihypertensive effects of protein hydrolysates are not always correlated, since the physiological transformations during digestion determine the bioavailability of these peptides and, as a result, their bioactivity. Moreover, it has also been demonstrated that the in vivo ACEI activity is not the only mechanism underlying the antihypertensive effects of some bioactive peptides. In this sense, certain food bioactive peptides have shown direct effects on relaxation in vascular smooth muscle [24].

In a previous study, we observed that the administration of 5 mL/kg bw (body weight) of a chicken foot hydrolysate, Hpp11, exhibited an antihypertensive effect in spontaneously hypertensive rats (SHR), which is considered to be a model for human essential HTN [25]. Thus, considering that 100 mg/kg bw dose of other protein hydrolysates showed substantial antihypertensive effects [26–28], doses lower than the antihypertensive demonstrated dose of 100 mg/kg bw; 85, 55 and 25 mg/kg bw of Hpp11, were administered to SHR. Therefore, the aims of the present study were to evaluate the most effective Hpp11 dose to obtain a significant antihypertensive effect in SHR and to investigate the mechanisms underlying the Hpp11 antihypertensive effect. Moreover, the effect of Hpp11 on the arterial SBP of Wistar-Kyoto (WKY) rats, the normotensive control for SHR, was also studied to rule out a potential hypotensive effect of this hydrolysate.

2. Materials and Methods

2.1. Chemicals and Reagents

Chicken feet from *Gallus gallus domesticus* were provided by a local farm (Granja Gaià, La Riera de Gaià, Spain). Protamex® (Novozymes, Bagsværd, Denmark) (EC 3.4.21.62 and 3.4.24.28, 1.5 AU/g from *Bacillus licheniformis* and *Bacillus amyloliquefaciens*), was kindly provided by Novozymes (Bagsværd, Denmark). ACE (angiotensin-converting enzyme, EC 3.4.15.1), *N*-Hippuryl-His-Leu (Hip-His-Leu), Captopril (PubChem CID: 44093) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and o-aminobenzoylglicil-p-nitrofenilalanilprolina (o-Abz-Gly-p-Phe(NO_2)-Pro-OH, PubChem CID: 128860) was provided by Bachem Feinchemikalien (Bubendorf, Switzerland). Acetylcholine (PubChem CID: 187), methoxamine hydrochloride (PubChem CID: 6081) and heparin heparin (PubChem CID: 772) were purchased from Sigma-Aldrich (Madrid, Spain). All other chemical solvents used were of analytical grade.

2.2. Chicken Foot Hydrolysate Hpp11: Obtainment and Characterisation

Chicken feet were mechanically disrupted, and sieves were utilized to obtain the protein hydrolysate, Hpp11 [18]. Protein powder with a size ≤2 mm was suspended in distilled water (20 mg/mL, *w*/*v*) and incubated for 1.5 h in a water bath set at 100 °C at 100 rpm. Subsequently, an enzymatic solution, Protamex®, was added at a final concentration of 2.67 µg/mL (enzyme/substrate ratio, 0.4 AU/g protein). Hydrolysis was carried out at 50 °C for 2 h at pH 7.0 in a MaxQ Orbital Shaker Thermo Scientific (Thermo Fisher Scientific, Waltham, MA, USA). At the end of the reaction, the enzyme was heat inactivated (80 °C, 10 min) in a water bath. Then, hydrolysate was centrifuged at 10,000× *g* for 20 min at 4 °C, and the supernatant was filtered through a 0.45 µm membrane, collected and lyophilized. Hpp11 was reconstituted in water to carry out the following experiments.

Hpp11 was characterized before its administration to SHR. Hpp11 protein content was estimated by the determination of total nitrogen compounds content of Hpp11 by the Kjeldahl method, multiplying the determined nitrogen content by 6.25 and the humidity determination was carried out following the AOAC official methods [29]. The degree of hydrolysis was determined by the TNBS method according to Adler-Nissen (1979) [30], in which free α-amino groups were determined. The Hpp11 ACEI activity was determined according to Quirós et al. [31]. The fluorescence measurements were performed after 30 min in a multi-scan microplate fluorimeter (FLUOstar optima, BMG Labtech, Offeuburg, Germany). The excitation and emission wavelengths were 360 and 400 nm, respectively. The software used to process the data was FLUOstar control (version 1.32 R2, BMG Labtech, Offeuburg, Germany).

The inhibition pattern of Hpp11 on the ACE substrate o-Abz-Gly-p-Phe(NO_2)-Pro-OH was assayed at the following concentrations: 7.2, 3.6, 1.8, 0.9, 0.45, 0.23 and 0 mM. The inhibition kinetics of ACE in the presence of Hpp11 was determined by Lineweaver–Burk plots [30].

All the analyses were performed in triplicate.

2.3. Experimental Procedure in the SHR and WKY Rats

Male SHR and WKY rats (17–20-week-old, weighing 300–350 g) were obtained from Charles River Laboratories España S.A. (Barcelona, Spain). The animals were housed at a temperature of 23 °C with 12 h light/dark cycles and consumed tap water and a standard diet (A04 Panlab, Barcelona, Spain) ad libitum during the experiments.

Different doses of the hydrolysate (25, 55 and 85 mg/kg bw) or a single dose of Hpp11 (55 mg/kg bw) were administered by gastric intubation to SHR or WKY rats, respectively, between 9 and 10 am. Tap water was used as a negative control for the SHR and WKY rats, and 50 mg/kg Captopril dissolved in tap water was given as a positive control to the SHR. The total volume of water, Captopril or Hpp11 orally administered to the rats was between 1.5 and 2 mL.

The systolic blood pressure (SBP) was recorded in the rats by the tail-cuff method [32] before and 2, 4, 6, 8, 24 and 48 h post-administration. Before the measurement, the animals were kept at 38 °C for 10 min in order to detect the pulsations of the tail artery. Changes in the SBP were expressed as the differences between the mean values of these variables before and after the administration of the treatment. To minimize stress-induced variations in BP, all measurements were taken by the same person, in the same peaceful environment. Moreover, before starting the experiments, we established a 2-week training period for the rats to become accustomed to the procedure. Data are expressed as the mean values ± standard error of the means (SEM) for a minimum of six experiments.

Additionally, twelve 20–23-week-old SHR weighing 350–380 g were administered Hpp11 at 55 mg/kg bw or water to determine the plasmatic ACE activity. The Hpp11 and water were orally administered by gastric intubation between 9 and 10 am. Blood samples were collected at 6 h post-administration via the saphenous vein using heparin vials. The samples were centrifuged at 2000× *g* for 15 min at 4 °C to obtain plasma. The procedure that was used to determine the plasmatic ACE activity is described below.

2.4. Determination of the Plasmatic ACE Activity

The plasmatic ACE activity was performed by a fluorometric method reported by Miguel et al. [28]. The measurements were performed in a multi-scan microplate fluorimeter (FLUOstar optima, BMG Labtech) at 37 °C and 350 nm excitation with 520 nm emission filters. ACE at different concentrations was added to each plate to obtain a calibration curve. ACE activity was expressed as the mean ± SEM mU ACE/mL of plasma for at least three replicates.

2.5. Experiments in Aorta Rings

Male 17–22-week-old, non-treated Sprague-Dawley (SD) rats weighing 250–300 g were sacrificed by decapitation. The thoracic aorta was excised from the animal's thorax, and excess fat and connective tissue were removed. To obtain the aorta preparations, the tissue was placed in a dissecting dish containing Krebs-Henseleit solution (NaCl, 118 mM: KCl, 4.7 mM; $CaCl_2$, 2.5 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 25 mM; and glucose, 10.0 mM) and cut into 3–4 mm rings. The aorta rings were mounted between two steel hooks in organ baths containing Krebs-Henseleit solution at 37 °C and continuously bubbled with a 95% O_2 and 5% CO_2 mixture, which gave a pH of 7.4. An optimal tension of 2 g was applied to all the aortic rings and adjusted every 15 min during the 60–90 min equilibration period, before adding the assayed compounds. The isometric tension was recorded by using an isometric force displacement transducer connected to an acquisition system (Protos 5, Panlab, Barcelona, Spain). After the equilibration period, 80 mM KCl was added to verify their functionality, and when the contraction had reached the steady state (approximately 15 min after the administration), the preparations were washed until the basal tension was recovered. The rings were then exposed to 10^{-5} M methoxamine, and when the contraction had reached the steady state, 10 µL Hpp11 was added to the organ bath at cumulative doses to reach concentrations between 0.01 mg/mL and 5 mg/mL. Water (10 µL) was used as negative control. The relaxant responses were expressed as a percentage of the pre-contraction induced by methoxamine, which was considered 100 percent. Results are expressed as the means ± SEM for at least eight experiments using aorta rings extracted from different animals. Concentration–response curves were fitted to the logistic equation, and statistical analysis was performed to compare concentration–response curves.

All the animal protocols followed in this study were approved by the Bioethical Committee of the Universitat Rovira i Virgili (European Comission Directive 86/609) and the Spanish Royal Decree 223/1988.

2.6. Statistical Analysis

The results are expressed as the mean ± SEM. Differences between the Hpp11 doses in the SHR were analysed by a two-way analysis of variance (ANOVA), and the Hpp11 effect on the WKY rats was analysed by a one-way analysis of variance (one-way ANOVA). To analyse differences between multiple independent groups, one-way analysis of variance followed by Tukey's or Dunnet's T3 post hoc test were used when required. The plasmatic ACE results were analysed by Student's *t*-test. Differences between concentration–response curves were analysed by two-way analysis of variance (two-way ANOVA). All the analyses were performed using IBM SPSS Statistics (SPSS, Chicago, IL, USA). Outliers were determined by using Grubbs' test. Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1. Chicken Foot Hydrolysate Hpp11

Chicken foot hydrolysate, Hpp11, was characterized before the in vivo experiments. According to ACE inhibition, it was observed that the IC_{50} value (concentration of the hydrolysate needed to inhibit 50% of the original ACE activity) of Hpp11 was 0.027 mg/mL. Table 1 shows the results of the Hpp11

determination of protein content, expressed as the total nitrogen compounds content, the humidity, the ash content, the degree of hydrolysis and the ACE inhibition as percentage.

Table 1. Protein content, humidity, ash content and degree of hydrolysis of the chicken foot hydrolysate Hpp11.

Determinations	(%)
Total protein content [a]	0.67
Humidity	98.93
Ash content [b]	0.17
Degree of hydrolysis [c]	18.85
ACE inhibition [d]	95.11

[a] Protein content was estimated by the measure of total nitrogen compounds content measured by the Kjeldahl method, expressed as *w*/*v*; [b] Ash content is expressed as g ash/100 g of product; [c] Degree of hydrolysis was measured by the TNBS method in which free α-amino groups were determined. The data shown are mean values of each parameter for at least two different hydrolysates assayed under the same conditions. [d] Angiotensin-converting enzyme (ACE) inhibitory activity (%).

3.2. Hpp11 In Vitro Inhibition Pattern on ACE

Figure 1 shows the Lineweaver-Burk plot of ACE activity in presence of Hpp11. Considering that the Lineweaver-Burk plot obtained by changing the substrate concentration intersects with the *y*-axis, the inhibition of ACE by Hpp11 corresponds to competitive inhibition.

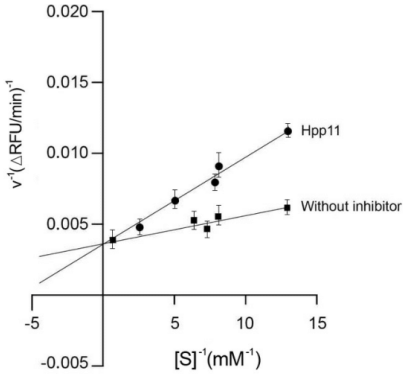

Figure 1. Lineweaver-Burk plot of angiotensin-converting enzyme (ACE) inhibition by chicken foot hydolysate Hpp11 and the control (without inhibitor). The Hpp11 effects at varying concentrations of ACE substrate (0–7.2 mM).

3.3. Effect of Different Doses of Hpp11 on Blood Pressure in Hypertensive and Normotensive Rats

Figure 2 shows the effect of three different doses of Hpp11 in SHR. Initial values of the SBP in the SHR were 195.9 ± 3.15 mmHg. As expected, the rats that only received water did not change their SBP values. In contrast, administration of Captopril (50 mg/kg bw) caused a clear decrease in the SBP, reaching the maximum decrease at 6 h post-administration. Regarding the hydrolysate, oral administration of 25 mg/kg bw did not produce an antihypertensive effect in the SHR. However, Hpp11 at 55 or 85 mg/kg bw resulted in a significant decrease in the SBP, reaching the maximum decrease at 6 h post-administration (−26.33 ± 2.1 and −30.45 ± 1.65 mmHg, respectively). At this time, the SBP decreases produced by both doses were similar to the decreases caused by Captopril. In fact, no significant differences between the decrease in BP produced by both Hpp11 doses was observed; however, the 55 mg/kg bw dose produced a more sustained antihypertensive effect than

85 mg/kg bw, showing a similar behaviour when compared to Captopril. In this sense, the SBP initial values were recovered 24 or 8 h post-administration at 55 and 85 mg/kg bw, respectively.

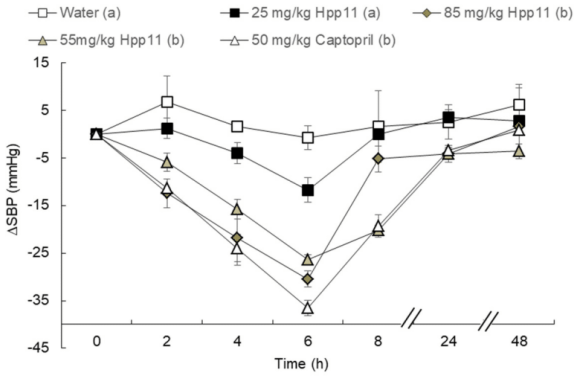

Figure 2. Decrease in the systolic blood pressure (SBP) in spontaneously hypertensive rats after the administration of water, Captopril (50 mg/kg bw) or different doses of chicken foot hydrolysate Hpp11: 25 mg/kg bw, 55 mg/kg bw and 85 mg/kg bw. Data are expressed as the mean ± SEM. All of the experimental groups include a minimum of six animals. Different letters represent significant differences ($p < 0.05$). p was estimated by two-way ANOVA.

In addition, oral administration of Hpp11 at a single dose of 55 mg/kg bw did not modify the arterial SBP in the normotensive WKY rats during the experiment (Figure 3). In fact, the SBP from the treated group showed similar values as the group administered with water.

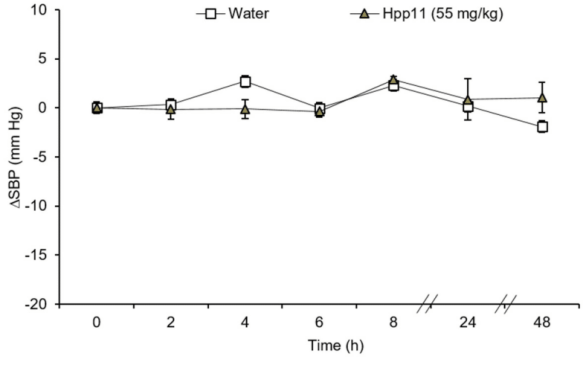

Figure 3. Decrease in the systolic blood pressure (SBP) in Wistar–Kyoto rats after administration of water or 55 mg/kg bw of chicken foot hydrolysate Hpp11. Data are expressed as the mean ± SEM. Both experimental groups have a minimum of six animals. No significant differences were observed.

3.4. Mechanisms Involved in the Antihypertensive Effect of Hpp11

The plasmatic ACE activity was measured in the rats administered 55 mg/kg bw of Hpp11 or water, 6 h post-administration. A reduction of 21% in the plasmatic ACE activity was found in the group administered Hpp11, being significantly lower than plasmatic ACE activity presented by the group administered water (Figure 4).

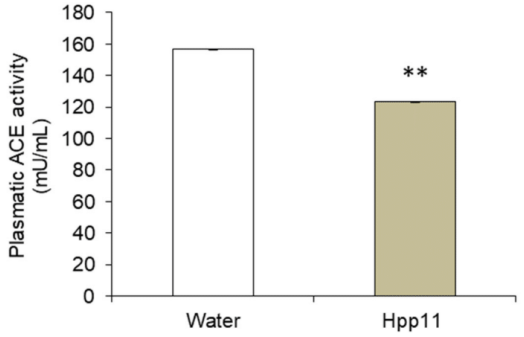

Figure 4. The plasmatic angiotensin-converting enzyme activity (ACE) in spontaneously hypertensive rats, 6 h after administration of 55 mg/kg chicken foot hydrolysate Hpp11 or water. Data are expressed as the mean ± SEM. The experimental groups include a minimum of six animals. The asterisks indicate differences between groups at $p < 0.01$ (**). p was calculated by Student's t-test.

In addition, to evaluate the existence of other mechanisms of antihypertensive activity in addition to ACE inhibition, the vascular effects of Hpp11 in aorta of SD rats was investigated. As demonstrated in Figure 5, Hpp11 did not produce relaxation in the aortic segments pre-contracted by methoxamine showing a similar effect to the control group.

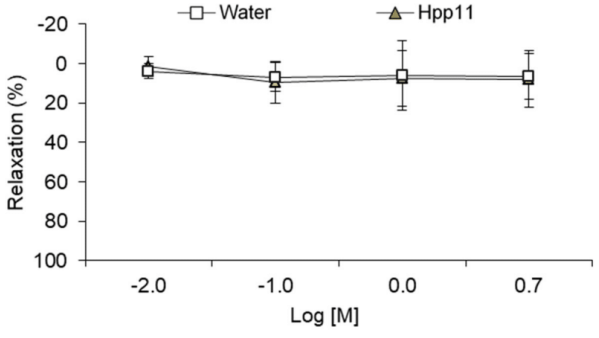

Figure 5. Cumulative concentration–response curves of chicken foot hydrolysate Hpp11 (0.01–5 M) in methoxamine pre-constricted aortic rings from Sprague-Dawley rats. Water was employed as a control, adding the same volume used to carry out the concentration-response curves of Hpp11. Data are mean values ± SEM. Both experimental groups have a minimum of six animals. No significant differences were observed between control group and Hpp11 group.

4. Discussion

The beneficial effects of bioactive peptides derived from hydrolyzed dietary proteins have been reported in many studies [33,34]. Some of these peptides demonstrate antihypertensive effects among other activities. Considering that commonly used antihypertensive drugs could present undesirable side effects, the interest in the use of protein hydrolysates to alleviate HTN has increased in recent years [35]. It is well known that chicken proteins are a good source of antihypertensive peptides. Onuh et al. demonstrated that peptides included in chicken skin protein hydrolysates were able to produce an antihypertensive effect after their administration to SHR [22]. Similar results were obtained from Saiga et al., postulating that chicken is a great source of bioactive peptides in part due to its high content of collagen [36], previously demonstrated to be a precursor of bioactive

peptides [37]. Considering this, in a previous study our group it was demonstrated that chicken foot proteins are a potential source of antihypertensive peptides [18]. In this previous study, chicken foot hydrolysates were obtained and one of the hydrolysates (Hpp11) exhibited a clear antihypertensive effect when administered at a dose of 5 mL/kg bw to the SHR as well as in vitro ACEI activity [18]. In this sense, protein hydrolysates administered at 100 mg/kg bw have been demonstrated to exert substantial antihypertensive effects [22,28]. Thus, in this study, three different lower doses 85, 55 and 25 mg/kg bw were administered to SHR to identify the dose with the maximum antihypertensive effect. Only the doses of 55 and 85 mg/kg bw exhibited the antihypertensive effect, reaching, in both cases, the maximum decrease at 6 h post-administration. Importantly, no differences between Hpp11 at 55 and 85 mg/kg bw and Captopril 50 mg/kg bw were found, suggesting the potential antihypertensive effect of this hydrolysate. In this sense, many studies reported the beneficial effects of protein hydrolysates in treating HTN conditions [21,22,38,39]; however, it is important to note that the doses used in this study are significantly lower than the doses reported for different protein-derived hydrolysates. Accordingly, the blood pressure-lowering effect exhibited by Hpp11 at 55 and 85 mg/kg bw (-26.33 ± 2.1 and -30.45 ± 1.65 mmHg, respectively) was similar to those reported by Miguel et al. for the antihypertensive hydrolysate from egg white. However, in this study, 100 mg/kg bw dose was required to observe this antihypertensive effect [28]. Interestingly, chicken-leg bone protein hydrolysate showed similar antihypertensive results (-26 mmgHg) being administered at a dose of 50 mg/kg bw [39], demonstrating the antihypertensive potential effect of chicken by-products. Nevertheless, the dose used to obtain this reduction in BP was 600 mg/kg bw [40], significantly higher than the dose of Hpp11 used in the present study. As mentioned before, both doses (55 and 85 mg/kg bw) presented similar antihypertensive effects, but it was only the 55 mg/kg bw dose that maintained the antihypertensive effect 8 h post-administration. The SBP from the Hpp11 treated groups at 55 and 85 mg/kg bw were completely restored 24 h post-administration. It is known that ACE inhibitory activity in vitro does not always correspond to an antihypertensive effect in vivo. This is mainly due to the bioavailability of the ACE inhibitory peptides after oral administration and the fact that peptides may influence blood pressure by mechanisms other than ACE inhibition. In vivo protein digestion could produce peptide modifications that could inactivate or activate antihypertensive peptides [41,42]. Considering this, in vivo assays are always required to demonstrate in vivo bioactivity of these protein hydrolysates able to inhibit ACE in vitro. These observations indicate that the ACE-inhibiting peptides in the 55 and 85 mg/kg bw Hpp11 were bioavailable either intact or in modified forms to exert short-term antihypertensive effects. Then, these peptides were rapidly metabolized into inactive products leading to the subsequent reduction in activity, especially for 85 mg/kg bw Hpp11, with no apparent antihypertensive by 8 h post-administration. A similar antihypertensive pattern was recently described by Udenigwe et al. using by-product hen meat protein hydrolysates [43]. These results showed that at relatively low doses, Hpp11 was able to reduce the SBP in the same manner as Captopril, demonstrating its potential applicability in HTN treatment. Moreover, the fact that relatively low doses are enough to obtain a potent antihypertensive effect increases its industrial value.

It is also important to point out that the administration of Hpp11 to normotensive WKY rats did not modify the BP of these animals. This indicates that the effect of Hpp11 is specific to the hypertensive condition. Therefore, these products could be used as functional foods without any risk in normotensive subjects.

One of the most common mechanisms likely involved in the BP-lowering effect of food peptides is ACE inhibition. Therefore, hydrolysate selection by their ACEI activity in vitro is a potential strategy for the selection of antihypertensive hydrolysates and peptides [6]. In fact, Hpp11 was selected by its great ability to inhibit ACE in vitro. However, in vitro ACEI activity does not always correspond to the same bioactivity in vivo because of the physiological transformations during protein digestion. Thus, plasmatic ACE activity in the SHR treated with 55 mg/kg bw was evaluated 6 h post-administration. ACE activity was significantly reduced in the Hpp11 treated group compared to the water treated

group. Similar results were reported after the administration of the ACE-inhibitory peptides contained in egg yolk [44] and soya protein [45].

Moreover, the inhibitory pattern of Hpp11 on in vitro ACE was studied. It was demonstrated that the peptides contained in the hydrolysate bind competitively at the active site of ACE to produce its inhibition. Quirós et al. [46] reported the same inhibition pattern for β-casein-peptides with antihypertensive properties. In this sense, it is well known that the most common mechanism of action of peptides in ACE inhibition is different from that of synthetic drugs. Generally, drugs indiscriminately block ACE and interfere with its activity, while ACE inhibitory peptides competitively block the binding of Ang I to ACE, thereby inhibiting the formation of Ang II [14,47]. Considering our results, Hpp11 produced its in vivo antihypertensive action by inhibiting the ACE activity, which suggests a possible reduction in Ang II release. However, many other systems different from RAAS can contribute to the control of BP. In this context, hydrolysates are complex mixtures of different peptides, and mechanisms of action other than ACE inhibition could be involved in their antihypertensive effect. In fact, it has been reported that antihypertensive food peptides can also have antioxidant, vasodilator, and/or opioid activities. In this sense, Sipola et al. [48] reported on the vasodilator-mediated antihypertensive effect of milk-derived peptides. Similar results were reported by Fujita et al., who reported that the antihypertensive effect of human casein-derived peptides was mediated by inducing relaxation in arteries [49]. To evaluate the potential Hpp11 vasodilator effect, aortic rings from SD rats were pre-contracted with methoxamine and then Hpp11 at increasing doses was administered. To evaluate the potential effects of different compounds on the vasculature, normotensive animals are used. SD rats are normotensive animals, such as WKY. However, SD rats are widely used to perform this type of study, considering that they offer higher response to contractile and relaxing agents than WKY, allowing it to demonstrate more clearly the effect of the tested compound [50].

Nevertheless, Hpp11 did not induce any relaxation in these preparations, showing similar behaviour to the control (water). However, it should be highlighted that SD rats are normotensive animals and Hpp11 may have different effects in the arteries of hypertensive animals. Future studies evaluating the Hpp11 effect on aortic rings from SHR could be performed to analyse the possible differences in their arterial responses to Hpp11. Moreover, the aorta is a conduit artery, and the resistance arteries determine the arterial BP more so than large vessels [51]. Therefore, we also suggest the use of resistance arteries for future studies to evaluate the Hpp11 vasodilator effect.

5. Conclusions

In this study, we demonstrated that the most effective antihypertensive dose of the chicken foot hydrolysate Hpp11 was 55 mg/kg after an acute administration. Our results indicate that Hpp11 produces its antihypertensive effect through the inhibition of ACE. Therefore, Hpp11 at low doses could be used as a functional food ingredient with potential therapeutic benefits in the prevention and treatment of HTN. Nevertheless, considering that HTN is a chronic pathology that requires chronic treatment, the evaluation of long-term administration of Hpp11 is necessary in animals and humans before the use of Hpp11 as an antihypertensive functional ingredient. Moreover, further studies are needed to determine the amino acid sequences present in Hpp11, responsible for the observed antihypertensive effect.

6. Patents

Patent application "Hydrolysates of chicken leg, their peptides and their uses": application number P201731065.

Author Contributions: Conceptualization, B.M. and F.I.B.; Formal analysis, A.M.-C., Z.P. and F.I.B.; Funding acquisition, B.M. and F.I.B.; Investigation, A.M.-C., Z.P. and F.I.B.; Methodology, A.M.-C., Z.P. and F.I.B.; Supervision, B.M., F.I.B. and A.A.; Writing—Original Draft, A.M.-C.; Writing—Review & Editing, B.M., F.I.B. and A.A.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Messerli, F.H.; Williams, B.; Ritz, E. Essential hypertension. *Lancet* **2007**, *370*, 591–603. [CrossRef]
2. Hedayati, S.; Elsayed, E.; Reilly, R. Non-pharmacological aspects of blood pressure management: What are the data? *Kidney Int.* **2011**, *79*, 1061–1070. [CrossRef] [PubMed]
3. Zhuang, Y.; Sun, L.; Zhang, Y.; Liu, G. Antihypertensive effect of long-term oral administration of jellyfish (*Rhopilema esculentum*) collagen peptides on renovascular hypertension. *Mar. Drugs* **2012**, *10*, 417–426. [CrossRef] [PubMed]
4. Cheng, F.Y.; Wan, T.C.; Liu, Y.T.; Chen, C.M.; Lin, L.C.; Sakata, R. Determination of angiotensin-I converting enzyme inhibitory peptides in chicken leg bone protein hydrolysate with alcalase. *Anim. Sci. J.* **2009**, *80*, 91–97. [CrossRef] [PubMed]
5. Sica, D.A. Angiotensin-converting enzyme inhibitors side effects—Physiologic and non-physiologic considerations. *J. Clin. Hypertens.* **2004**, *6*, 410–416. [CrossRef]
6. Margalef, M.; Bravo, F.I.; Arola-Arnal, A.; Muguerza, B. Natural angiotensin converting enzyme (ACE) inhibitors with antihypetensive properties. In *Natural Products Targeting Clinically Relevant Enzymes*; Andrade, P., Valentao, P., Pereira, D.M., Eds.; Wiley-VCH Verlag GmbH & Co.: Weinheim, Germany, 2017; pp. 45–67.
7. Iwaniak, A.; Minkiewicz, P.; Darewicz, M. Food-originating ACE inhibitors, including antihypertensive peptides, as preventive food components in blood pressure reduction. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 114–134. [CrossRef]
8. Chakrabarti, S.; Wu, J. Bioactive peptides on endothelial function. *Food Sci. Hum. Wellness* **2016**, *5*, 1–7. [CrossRef]
9. Fang, H.; Luo, M.; Sheng, Y.; Li, Z.; Wu, Y.; Liu, C. The antihypertensive effect of peptides: A novel alternative to drugs? *Peptides* **2008**, *29*, 1062–1071.
10. Martínez-Maqueda, D.; Miralles, B.; Recio, I.; Hernández-Ledesma, B. Antihypertensive peptides from food proteins: A review. *Food Funct.* **2012**, *3*, 350. [CrossRef] [PubMed]
11. Aleixandre, A.; Miguel, M.; Muguerza, B. Péptidos antihipertensivos derivados de proteínas de leche y huevo. *Nutr. Hosp.* **2008**, *23*, 313–318. [PubMed]
12. Huang, J.; Liu, Q.; Xue, B.; Chen, L.; Wang, Y.; Ou, S.; Peng, X. Angiotensin-I-converting enzyme inhibitory activities and in vivo Antihypertensive Effects of Sardine Protein Hydrolysate. *J. Food Sci.* **2016**, *81*, H2831–H2840. [CrossRef] [PubMed]
13. Slizyte, R.; Rommi, K.; Mozuraityte, R.; Eck, P.; Five, K.; Rustad, T. Bioactivities of fish protein hydrolysates from defatted salmon backbones. *Biotechnol. Rep.* **2016**, *11*, 99–109. [CrossRef] [PubMed]
14. Ahhmed, A.M.; Muguruma, M. A review of meat protein hydrolysates and hypertension. *Meat Sci.* **2010**, *86*, 110–118. [CrossRef] [PubMed]
15. Ketnawa, S.; Rawdkuen, S. Angiotensin converting enzyme inhibitory peptides from aquatic and their processing by-Products: A review. *Int. J. Sci. Innov. Discov.* **2013**, *2*, 185–199.
16. Karamaæ, M.; Flaczyk, E.; Wanasundara, P.K.J.P.D.; Amarowicz, R. Angiotensin I-converting enzyme (ACE) inhibitory activity of hydrolysates obtained from muscle food industry by-products—A short report. *Pol. J. Food Nutr. Sci.* **2005**, *14*, 133–137.
17. Mora, L.; Reig, M.; Toldrá, F. Bioactive peptides generated from meat industry by-products. *Food Res. Int.* **2014**, *65*, 344–349. [CrossRef]
18. Bravo, F.I.; Arola, L.; Muguerza, B. Procedure for obtaining a hydrolysate claw chicken leg with antihypertensive activity, and peptides obtained hydrolysate containing. Patent No. ES2606954B1, 11 December 2017.

19. Lasekan, A.; Abu Bakar, F.; Hashim, D. Potential of chicken by-products as sources of useful biological resources. *Waste Manag.* **2013**, *33*, 552–565. [CrossRef] [PubMed]
20. Toldrá, F.; Aristoy, M.C.; Mora, L.; Reig, M. Innovations in value-addition of edible meat by-products. *Meat Sci.* **2012**, *92*, 290–296. [CrossRef] [PubMed]
21. Onuh, J.O.; Girgih, A.T.; Aluko, R.E.; Aliani, M. Inhibitions of renin and angiotensin converting enzyme activities by enzymatic chicken skin protein hydrolysates. *Food Res. Int.* **2013**, *53*, 260–267. [CrossRef]
22. Onuh, J.O.; Girgih, A.T.; Malomo, S.A.; Aluko, R.E.; Aliani, M. Kinetics of in vitro renin and angiotensin converting enzyme inhibition by chicken skin protein hydrolysates and their blood pressure lowering effects in spontaneously hypertensive rats. *J. Funct. Foods* **2015**, *14*, 133–143. [CrossRef]
23. Onuh, J.O.; Girgih, A.T.; Nwachukwu, I.; Ievari-Shariati, S.; Raj, P.; Netticadan, T.; Aluko, R.E.; Aliani, M. A metabolomics approach for investigating urinary and plasma changes in spontaneously hypertensive rats (SHR) fed with chicken skin protein hydrolysates diets. *J. Funct. Foods* **2016**, *22*, 20–33. [CrossRef]
24. Majumder, K.; Wu, J. Molecular Targets of Antihypertensive Peptides: Understanding the mechanisms of action based on the pathophysiology of hypertension. *Int. J. Mol. Sci.* **2015**, *16*, 256–283. [CrossRef] [PubMed]
25. Miguel, M.; Manso, M.; Aleixandre, A.; Alonso, M.J.; Salaices, M.; Lopez-Fandino, R. Vascular effects, angiotensin I-converting enzyme (ACE)-inhibitory activity, and antihypertensive properties of peptides derived from egg white. *J. Agric. Food Chem.* **2007**, *55*, 10615–10621. [CrossRef] [PubMed]
26. Girgih, A.T.; Nwachukwu, I.D.; Onuh, J.O.; Malomo, S.A.; Aluko, R.E. Antihypertensive properties of a pea protein hydrolysate during short- and long-term oral administration to spontaneously hypertensive rats. *J. Food Sci.* **2016**, *81*, H1281–H1287. [CrossRef] [PubMed]
27. Lin, H.-C.; Alashi, A.M.; Aluko, R.E.; Sun Pan, B.; Chang, Y.-W. Antihypertensive properties of tilapia (*Oreochromis* spp.) frame and skin enzymatic protein hydrolysates. *Food Nutr. Res.* **2017**, *61*, 1391666. [CrossRef] [PubMed]
28. Miguel, M.; Alonso, M.J.; Salaices, M.; Aleixandre, A.; López-Fandiño, R. Antihypertensive, ACE-inhibitory and vasodilator properties of an egg white hydrolysate: Effect of a simulated intestinal digestion. *Food Chem.* **2007**, *104*, 163–168. [CrossRef]
29. Association of Official Analytical Chemists (AOAC) (Ed.) *Official Methods of Analysis*, 16th ed.; Association of Official Analytical Chemists: Arlington, VA, USA, 1995.
30. Adler-Nissen, J. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *J. Agric. Food Chem.* **1979**, *27*, 1256–1262. [CrossRef] [PubMed]
31. Quirós, A.; del Contreras, M.M.; Ramos, M.; Amigo, L.; Recio, I. Stability to gastrointestinal enzymes and structure–activity relationship of β-casein-peptides with antihypertensive properties. *Peptides* **2009**, *30*, 1848–1853. [CrossRef] [PubMed]
32. Buñag, R.D. Validation in awake rats of a tail-cuff method for measuring systolic pressure. *J. Appl. Physiol.* **1973**, *34*, 279–282. [CrossRef] [PubMed]
33. Yamamoto, N. Antihypertensive peptides derived from food proteins. *Biopolym. Pept. Sci. Sect.* **1997**, *43*, 129–134. [CrossRef]
34. Udenigwe, C.C.; Mohan, A. Mechanisms of food protein-derived antihypertensive peptides other than ACE inhibition. *J. Funct. Foods* **2014**, *8*, 45–52. [CrossRef]
35. Hou, Y.; Wu, Z.; Dai, Z.; Wang, G.; Wu, G. Protein hydrolysates in animal nutrition: Industrial production, bioactive peptides, and functional significance. *J. Anim. Sci. Biotechnol.* **2017**, *8*, 1–13. [CrossRef] [PubMed]
36. Saiga, A.; Iwai, K.; Hayakawa, T.; Takahata, Y.; Kitamura, S.; Nishimura, T.; Morimatsu, F. Angiotensin I-converting enzyme-inhibitory peptides obtained from chicken collagen hydrolysate. *J. Agric. Food Chem.* **2008**, *56*, 9586–9591. [CrossRef] [PubMed]
37. Fu, Y.; Therkildsen, M.; Aluko, R.E.; Lametsch, R. Exploration of collagen recovered from animal by-products as a precursor of bioactive peptides: Successes and challenges. *Crit. Rev. Food Sci. Nutr.* **2018**, *8398*, 1–17. [CrossRef] [PubMed]
38. Balti, R.; Bougatef, A.; Ali, N.E.H.; Zekri, D.; Barkia, A.; Nasri, M. Influence of degree of hydrolysis on functional properties and angiotensin I-converting enzyme-inhibitory activity of protein hydrolysates from cuttlefish (*Sepia officinalis*) by-products. *J. Sci. Food Agric.* **2010**, *90*, 2006–2014. [CrossRef] [PubMed]
39. Cheng, F.Y.; Wan, T.C.; Liu, Y.T.; Lai, K.M.; Lin, L.C.; Sakata, R. A study of in vivo antihypertensive properties of enzymatic hydrolysate from chicken leg bone protein. *Anim. Sci. J.* **2008**, *79*, 614–619. [CrossRef]

40. Li, G.-H.; Qu, M.-R.; Wan, J.-Z.; You, J.-M. Antihypertensive effect of rice protein hydrolysate with in vitro angiotensin I-converting enzyme inhibitory activity in spontaneously hypertensive rats. *Asia Pac. J. Clin. Nutr.* **2007**, *16* (Suppl. 1), 275–280.
41. Vermeirssen, V.; Van Camp, J.; Verstraete, W. Bioavailability of angiotensin I converting enzyme inhibitory peptides. *Br. J. Nutr.* **2004**, *92*, 357. [CrossRef] [PubMed]
42. Jao, C.-L.; Huang, S.-L.; Hsu, K.-C. Angiotensin I-converting enzyme inhibitory peptides: Inhibition mode, bioavailability, and antihypertensive effects. *BioMedicine* **2012**, *2*, 130–136. [CrossRef]
43. Udenigwe, C.C.; Girgih, A.T.; Mohan, A.; Gong, M.; Malomo, S.A.; Aluko, R.E. Antihypertensive and bovine plasma oxidation-inhibitory activities of spent hen meat protein hydrolysates. *J. Food Biochem.* **2017**, *41*, 1–8. [CrossRef]
44. Yoshii, H.; Tachi, N.; Ohba, R.; Sakamura, O.; Takeyama, H.; Itani, T. Antihypertensive effect of ACE inhibitory oligopeptides from chicken egg yolks. *Comp. Biochem. Physiol. C* **2001**, *128*, 27–33. [CrossRef]
45. Yang, H.-Y.; Yang, S.-C.; Chen, J.-R.; Tzeng, Y.-H.; Han, B.-C. Soyabean protein hydrolysate prevents the development of hypertension in spontaneously hypertensive rats. *Br. J. Nutr.* **2004**, *92*, 507. [CrossRef] [PubMed]
46. Quirós, A.; Ramos, M.; Muguerza, B.; Delgado, M.A.; Miguel, M.; Aleixandre, A.; Recio, I. Identification of novel antihypertensive peptides in milk fermented with *Enterococcus faecalis*. *Int. Dairy J.* **2007**, *17*, 33–41. [CrossRef]
47. Alderman, C.P. Adverse effects of the angiotensin-converting enzyme inhibitors. *Ann Pharmacoter.* **1996**, *30*, 55–61. [CrossRef] [PubMed]
48. Sipola, M.; Finckenberg, P.; Vapaatalo, H.; Pihlanto-Leppälä, A.; Korhonen, H.; Korpela, R.; Nurminen, M.-L. Alpha-lactorphin and beta-lactorphin improve arterial function in spontaneously hypertensive rats. *Life Sci.* **2002**, *71*, 1245–1253. [CrossRef]
49. Fujita, H.; Suganuma, H.; Usui, H.; Kurahashi, K.; Nakagiri, R.; Sasaki, R.; Yoshikawa, M. Vasorelaxation by casomokinin L, a derivative of β-casomorphin and casoxin D, is mediated by NK1receptor. *Peptides* **1996**, *17*, 635–639. [CrossRef]
50. Rahmani, M.A.; DeGray, G.; David, V.; Ampy, F.R.; Jones, L. Comparison of calcium import as a function of contraction in the aortic smooth muscle of Sprague-Dawley, Wistar Kyoto and spontaneously hypertensive rats. *Front. Biosci.* **1999**, *4*, D408–D415. [PubMed]
51. Pons, Z.; Arola, L. Involvement of nitric oxide and prostacyclin in the antihypertensive effect of low-molecular-weight procyanidin rich grape seed extract in male spontaneously hypertensive rats. *J. Funct. Foods* **2014**, *6*, 419–427.

CHAPTER 3

To evaluate the effect of long-term administration of chicken foot hydrolysate Hpp11 in diet-induced hypertension

MANUSCRIPT 3

Objective

To investigate the long-term administration effect of chicken foot hydrolysate Hpp11 in diet-induced hypertension and to elucidate the molecular mechanisms involved in its chronic antihypertensive effect.

Long-term administration of protein hydrolysate from chicken feet induces antihypertensive effect and confers vasoprotective pattern in diet-induced hypertensive rats

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Keywords: Bioactive peptides; cafeteria diet; metabolic syndrome; endothelial function; protein hydrolysate

Chemical compound studied in this article: Captopril (PubChem CID: 44093) and heparin (PubChem CID: 772).

Abbreviations: **ACE,** angiotensin-converting enzyme; **BP**, blood pressure; **SBP**, systolic blood pressure; **CAF,** cafeteria; **HTN**, hypertension; **GSH**, reduced glutathione; **BW**, body weight; **MetS**, metabolic syndrome

Abstract

The aim of this study was to evaluate the effect of long-term administration of the protein hydrolysate from chicken feet Hpp11 in diet-induced hypertensive rats. Rats were fed standard or cafeteria (CAF) diets for 12 weeks, and during the final 3 weeks, CAF animals were administered vehicle, Captopril or Hpp11. Systolic blood pressure (SBP) was recorded weekly. Body weight and plasmatic lipid and glucose levels were determined. Moreover, liver-reduced glutathione (GSH) levels, plasmatic angiotensin converting enzyme (ACE) activity and the endothelial gene expression of eNOS, KLF-2, Sirt-1, NOX4 and ET-1 were studied. A definite decrease in SBP was recorded in the rats that were administered Hpp11. Hpp11 downregulated gene expression of the vasoconstrictor ET-1 and induced the upregulation of NOX4 and NO enhancer Sirt-1, suggesting the vasoprotective effect of Hpp11. Moreover, Hp11 increased hepatic GSH. The present findings suggest that Hpp11 is a good candidate to ameliorate diet-induced hypertension.

1. Introduction

Hypertension (HTN) is a common risk factor for cardiovascular disease (CVD) and a major global public health problem. It affects nearly 45% of the world population and results in over ten million deaths annually [1]. Remarkably, HTN frequently occurs concurrently with other CVD risk factors related to lifestyle, such as obesity, dyslipidemia and impaired glucose tolerance (hyperglycemia), resulting in metabolic syndrome (MetS) [2]. Rats fed a cafeteria (CAF) diet receive highly palatable, energy dense and unhealthy human food *ad libitum,* while they have free access to standard (STD) chow diet and water. These animals are considered a robust model of human MetS, displaying increased body weight (BW), increased abdominal fat, hyperinsulinemia, hyperglycemia, and hepatic steatosis [3]. The development of HTN in CAF diet-fed rats has also been reported [4]. The treatment of MetS is still a medical challenge, considering the complexity of this multifactorial disease [5]. In this sense, interest in the research of new therapies for MetS treatment is increasing, and the use of bioactive natural ingredients has emerged as a possible alternative in the prevention and treatment of this disease.

The hydrolysis of food proteins is considered a potential source of peptides with different bioactivities [6]. Among all these bioactivities, highlights the capacity of different peptides to inhibit angiotensin-converting enzyme (ACE), key-enzyme in blood pressure (BP) control. Different protein sources have been reported to release antihypertensive peptides such as milk and egg [7] or meat muscle [8], among others. In this sense, the interest of proteins derived from food by-products for this purpose has increased [9, 10]. The use of food by-products allows revalorizing this waste materials, making the food and agricultural industries more environmentally friendly [11]. In this regard,

our group has used chicken foot, a poultry industry by-product, to obtain protein hydrolysates presenting antihypertensive effect after acute administration [12]. The involvement of ACE inhibition in the antihypertensive effect of one of the obtained hydrolysates (Hpp11), was confirmed 6 h after its administration in hypertensive rats [12]. However, HTN is a chronic pathology requiring chronic treatment; thus, the evaluation of the effect of chronic administration of Hpp11 is required to evaluate its potential as an antihypertensive agent. In addition, the involvement of other mechanisms in the benefits of Hpp11 hydrolysate on HTN has not already been elucidated.

Indeed, other mechanisms of action, beyond ACE inhibition, have been reported to explain antihypertensive effects of biologically active peptides from different food sources [13]. A reduction in BP by restoring the endothelium-derived factors production homeostasis and by reducing oxidative stress has been reported for some bioactive peptides [14]. Endothelial tissue regulates vascular tone and exerts finely tuned control over cardiovascular homeostasis, including nitric oxide (NO), one of the best characterized vasodilator endothelial factors, and endothelin-1 (ET-1), one of the strongest known vasoconstrictors [15]. NO is synthesized in the endothelial cells by an isoform of the enzyme NO synthase (eNOS) [16], which in turn is regulated by eNOS-promoter transcription factor Krüpple like factor 2 (KLF-2). KLF-2 can inhibit the expression of ET-1 [17], which is overexpressed in the vasculature in different hypertensive models [18]. KLF-2 activation is also regulated by Sirtuin-1 (Sirt-1), which is mainly known as an eNOS promoter (Pons, Margalef, Bravo, Arola-Arnal, & Muguerza, 2016). In turn, Sirt-1 promotes the inhibition of NADPH oxidase (NOX) activity, which is known to be a reactive oxygen species (ROS) producer [20].

Furthermore, besides the imbalance between the production of vasodilators and vasoconstrictors, an increase in oxidative stress due to the presence of different pathological conditions can also lead to the development of endothelial dysfunction and, as a result, can induce an increase in BP [21]. In this sense, it has been reported that some protein hydrolysates present antioxidant effects and reduce oxidative stress. Udenigwe et al. [22] reported that an increase in the production of reduced glutathione (GSH), an endogen antioxidant system, is one of the antioxidant mechanisms exerted by these hydrolysates.

Therefore, the principal aim of the present study was to evaluate the SBP-lowering effect caused by Hpp11 after 3 weeks of administration in an animal model of HTN associated with MetS. To reach this objective, CAF diet-fed rats were used in this study. Moreover, the molecular mechanisms underlying the long-term effects of Hpp11 were studied in these animals.

2. Materials and methods

2.1. Chemicals and reagents

Chicken feet from *Gallus gallus domesticus* were provided by a local farm (Granja Gaià, La Riera de Gaià, Spain). Protamex® (EC 3.4.21.62 and 3.4.24.28, 1.5 AU/g from Bacillus licheniformis and Bacillus amyloliquefaciens), was kindly provided by Novozymes (Lyngby, Denmark). ACE (angiotensin converting enzyme, EC 3.4.15.1), N-Hippuryl-His-Leu (Hip-His-Leu), monochlorobimane and glutathione-S transferase from horse liver were purchased from Sigma-Aldrich (Madrid, Spain). Captopril was provided by Santa Cruz Biotechnology

(Dallas, TX, United States). All other chemical solvents used were of analytical grade.

2.2. Preparation and characterization of the chicken foot hydrolysate Hpp11

Hpp11 was obtained following the method described by Bravo et al. [23]. Briefly, chicken feet powder mixed with distilled water was pretreated for 1.5 h in a water bath set at 100 °C, 100 rpm. Subsequently, an enzymatic solution of Protamex® was added at a final concentration of 2.67 µg/mL (enzyme/substrate ratio, 0.4 AU/g protein). Hydrolysis was carried out at 50 °C for 2 h at pH 7.0 in a MaxQ Orbital Shaker Thermo Scientific (Thermo Fisher Scientific, USA). At the end of reaction, the enzyme was heat inactivated (80 °C, 10 min) in a water bath. Then, hydrolysate was centrifuged at 10,000×g for 20 min at 4 °C, and supernatant filtered through a 0.45 µm membrane was collected and lyophilized.

Total protein content was determined by the Kjeldahl method according to AOAC methods [24] (total nitrogen content was multiplied by 6.25). The degree of hydrolysis (D_H) was obtained following the TNBS method according to Adler-Nissen [25], in which free α-amino groups were determined. A leucine calibration curve was used. In addition, amino acid profile of the hydrolysate was determinate in this hydrolysate in duplicate. The identification and quantification of the amino acid content was performed by following the method described by Dahl-Lassen et al. [26] with some modifications. Thus, an acidic hydrolysis using 6 N HCl, under increasing temperatures until 150 ° C for 1.5 h, followed by basic hydrolysis using 4.2 N NaOH under the same conditions used in the

acidic hydrolysis. Then, samples were analyzed by UHPLC-QToF/MS to identify and quantify amino acid profile in Hpp11.

ACEI activity was determined according to Guerrero et al. [27] with some modifications. ACE working solution was diluted with 0.15 M Tris buffer (pH 8.3) containing 0.1 µM $ZnCl_2$ with 0.04 U/mL of enzyme in the final reaction solution. A total of 40 µL of sample, distilled water or this enzyme working solution were added to each microtiter-plate well and were adjusted to 80 µL by adding distilled water if it was necessary. The enzymatic reaction started by adding 160 µL of 0.45 mM o-Abz-Gly-p-Phe(NO_2)-Pro-OH dissolved in 150 mM Tris-base buffer (pH 8.3), containing 1.125 M NaCl, and the mixture was incubated at 37 °C. The sample fluorescence was recorded at 30 min using a multiscan microplate fluorimeter (FLUOstar optima, BMG Labtech, Offeuburg, Germany). Excitation and emission wavelengths were 360 and 400 nm, respectively. The software used to process the data were FLUOstar control (version 1.32 R2, BMG Labtech). The ACEI activity of Hpp11 was determined as the IC_{50} (µg of Hpp11 lyophilized). The IC_{50} is the concentration of each hydrolysate (µg of Hpp11 lyophilized) that reduces the enzyme activity by half. The determination of ACEI activity was performed at least in duplicate.

2.3 Experimental procedure in rats

Five-week-old male normotensive Wistar rats, Crl:WI were obtained from Charles River Laboratories (Barcelona, Spain). The rats were maintained at 22 °C under 12-h light/12-h dark conditions. They received STD chow Panlab A04 (Panlab, Barcelona, Spain) and tap water *ad libitum* during the quarantine period (10 days). After that, rats were divided into two dietary groups: the STD diet-fed rats, which were fed *ad libitum* STD chow Panlab A04 (Panlab, Barcelona, Spain)

and tap water, or CAF diet-fed rats, which were fed fresh CAF diet in addition to the STD diet and tap water. CAF diet consisted of biscuits with paté, biscuits with cheese, semi cured cheese, bacon, ensaïmada (sweetened pastry), carrots and milk with sucrose 20% (*w/v*) daily. The composition of the CAF diet was 35% fat, 51% carbohydrates, 14% protein and 0.2% Na, whereas STD diet was 4% fat, 76% carbohydrates, 20% protein with 0.3% Na [19]. The different diets were administered for 12 weeks. At the 9th week of the experiment, STD diet-fed rats group were orally administered sweetened milk, containing 20% sucrose, as vehicle (VH) (n=6 per group; STD+VH). CAF diet-fed rats were divided into 3 groups (n=6 per group) and were orally administered VH (CAF+VH), VH containing 50 mg/kg body weight (bw) of Captopril (CAF+Captopril) or VH containing 55 mg/kg bw of Hpp11 (CAF+Hpp11). All compounds were administered daily in a volume of 1 mL between 08.00 and 09.00 h for 3 weeks. Systolic blood pressure (SBP) was measured weekly during all the experiments by the same person to reduce stress-induced variations. SBP was recorded by the tail-cuff method [28] at 3 h post administration.

At the end of the experiment, the rats were fasted for 3 h after the oral administration, body weight (BW) was recorded, and then animals were killed by live decapitation. Total animal blood was collected into tubes containing lithium heparin (Deltalab, Barcelona, Spain) as an anticoagulant. The aorta and liver were excised, cleaned and immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Plasma was obtained after blood centrifugation at 1500×g for 15 min at 4 °C and stored at -80 °C until their use. The complete experimental design is schematized in Figure 1.

The Animal Ethics Committee of University Rovira i Virgili approved all procedures (reference number 7959 by Generalitat de Catalunya). All

of the above mentioned experiments were performed as authorized (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food, Madrid, Spain).

2.4 Biochemical plasma determinations

Plasma total cholesterol (TC), triglycerides (TG) and glucose were measured using enzymatic colorimetric kits following the manufacturer's protocols (QCA, Barcelona, Spain).

Plasmatic ACE was measured following the method reported by Pons et al. [4]. Briefly, triplicate plasma (3 µL) aliquots were incubated for 15–90 min at 37 °C with 40 µL of ACE substrate 5 mM Hip-His-Leu (Sigma) assay buffer. Then, 190 µL of 0.35N HCl were added to stop the reaction. The generated product, His-Leu, was measured fluorometrically following a 10-min incubation with 100 µL of 2% o-phathaldialdehyde in methanol. The measurements were performed in multiscan microplate fluorimeter FLUOstar optima (BMG Labtech) at 37 °C, using 350 nm excitation and 520 nm emission filters. Commercial ACE at different concentrations was used to obtain a calibration curve. ACE activity was expressed as the mean (mU ACE/mL of plasma) ± standard error of the mean (SEM) for at least three replicates.

2.5 Reduced glutathione assay

The GSH assay was performed in liver following the monochlorobimane fluorometric method [29]. To evaluate GSH levels, 90 µL of homogenized supernatant from the liver was mixed with monochlorobimane (100 mM) and 10 µL of the catalyst, glutathione S-transferase solution (1 U/mL). The levels of GSH were quantified using a multiscan microplate fluorimeter FLUOstar optima (BMG Labtech)

and were expressed as the mean (µmol of reduced glutathione/g tissue protein) ± SEM for at least three replicates.

2.6 RNA extraction and mRNA quantification by real-time qPCR

RNA extraction was performed in homogenized thoracic aorta using an RNeasy Mini Kit following the manufacturer's protocols (Qiagen, Barcelona, Spain). Total extracted RNA was quantified in a Nanodrop 100 Spectrophotometer (Thermo Scientific). mRNA retrotranscription was carried out by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain). Quantitative PCR amplification and detection were performed in the CFX96 Touch Real Time PCR System (Bio-Rad, Barcelona, Spain) using 96-well plates and SYBR PCR Premix Reagent Ex Taq™ (Takara, Barcelona, Spain) following the commercial protocol.

Relative mRNA levels of KLF-2, eNOS, Sirt-1, NOX4 and ET-1 were analyzed by real-time PCR using peptidylprolyl isomerase A (PPIA) as the housekeeping gene. Primers used for the different genes are shown in Table 2 and were obtained from Biomers (Söflinger, Germany). Primer specificity was verified by melting curve analysis, and the amplicon size was verified by 3% agarose gel electrophoresis. The efficiency of qPCR was calculated by evaluating a 2-fold dilution series of aortic cDNA and was calculated by $E = 10^{(1/slope)}$. The results were expressed as the logarithm of the cDNA concentration *vs* the obtained Ct. Relative expression was calculated by dividing the E^{Ct} of the studied gene by the E^{Ct} of the housekeeping gene used as the control and then divided by the value of the control situation, STD+VH group gene expression. Each sample was analyzed in triplicate.

2.7 Statistical analysis

The results were expressed as the mean ± SEM of six animals per group. The data were analyzed by one-way ANOVA or two-way ANOVA (Tukey's test) using IBM SPSS Statistics 20.0 software (SPSS, Inc, Chicago, IL, USA). Outliers were identified and eliminated by using Grubbs' test. The comparisons were considered significant at $p < 0.05$.

3. Results

3.1 Hpp11 characterization

Hpp11 was produced and characterized before its administration to CAF-diet rats. The Hpp11 protein content was 62.62 ± 0.00 % (w/w), the D_H was 19.62 ± 0.76 %. The amino acid profile, expressed as mg of amino acid residue per g of lyophilized chicken foot hydrolysate, showed that the most abundant amino acid residues Leucine (41.07 ± 1.26 mg/g), Isoleucine (28.18 ± 0.85 mg/g) and Proline (22.20 mg/g ± 0.53 mg/g) (Table 1). The ACE inhibitory activity, expressed as the IC_{50}, was 8.89 ± 0.93 µg of Hpp11.

3.2 Effect of Hpp11 on blood pressure

Figure 2 shows the changes in SBP in the CAF diet-fed rats administered VH, Captopril (50 mg/kg bw) or Hpp11 (55 mg/kg bw) for 3 weeks. The rats fed the CAF diet developed hypertension between the 6^{th} and 8^{th} week of diet consumption, reaching values of 142 ± 2 mmHg for SBP. At the 9^{th} week of diet consumption, supplementation of the CAF diet with VH, Captopril or Hpp11 was carried out. It was observed that while SBP from the CAF+VH group increased as the experiment progressed, reaching values of 149 ± 5 mmHg, the groups administered Captopril (CAF+Captoril) or Hpp11 (CAF+Hpp11) presented a significant decrease in SBP, showing values of 114 ± 3 and

131 ± 1 mmHg, respectively, at the end of the experiment. It is important to mention that since the first week of administration, Hpp11 was able to reduce SBP, showing a similar decrease to that produced by Captopril (-9 ± 1 and -10 ± 2 mmHg, respectively). The Hpp11-induced decrease in SBP was sustained for the three weeks of supplementation, showing that the values of this group were close to 130 mmHg during the three weeks of treatment. However, Captopril produced a more pronounced decrease in SBP that was able to restore initial values of SBP (117 ± 2 mmHg) at the end of the experiment.

3.3 Effect of Hpp11 on other cardiometabolic risk factors associated with the MetS

BW and the plasmatic TG, cholesterol and glucose values at the end of the experiment are shown in Table 1. The values of BW, plasmatic TG, plasmatic cholesterol and plasmatic glucose were significantly higher in the groups fed the CAF diet (CAF+VH, CAF+Captopril, CAF+Hpp11) than in the group fed standard diet (STD+VH).

3.4 Effects of Hpp11 on plasmatic ACE, the endogen antioxidant system and endothelial function-associated genes

Figure 3 shows the plasmatic ACE activity values. No differences were observed between the STD+VH and CAF groups administered VH and Hpp11. However, the CAF diet group administered Captopril presented significantly higher values in this parameter.

The Hpp11 effect was evaluated by the production of GSH in liver. It was observed that Hpp11 administration to CAF diet-fed rats produced a significant increase in GSH production compared to the CAF groups administered Captopril or VH and compared to the STD+VH group (Figure 4).

Finally, the Hpp11 effect on endothelial function-associated genes was studied in the aorta to determine whether the antihypertensive effect of long-term administration of Hpp11 could be related to the improvement of endothelial function (Figure 5). It was observed that Hpp11 significantly increased the mRNA expression of Sirt-1. KLF-2 was also increased in the Hpp11 group; however, there were no significant differences compared to STD+VH group or CAF+VH group. No differences in mRNA eNOS expression were observed in the group administered Hpp11 either.

Additionally, mRNA expression of NOX4 was upregulated in the CAF+Hpp11 group, while it was observed that Hpp11 produced a significant decrease in ET-1 expression.

4. Discussion

HTN is considered one of the most relevant risk factors for MetS, and its control clearly reduces the incidence of CVD [30, 31]. High BP is typically controlled by the chronic administration of antihypertensive drugs. Moreover, BW reduction and improving the plasmatic lipid profile have also been demonstrated to be essential for HTN control, especially when HTN is associated with MetS [32]. The chicken foot hydrolysate Hpp11 has recently been demonstrated to exhibit antihypertensive properties after acute administration in SHR [23]. Nevertheless, because HTN is a chronic pathology that requires chronic treatment [33], the evaluation of the potential antihypertensive effect of Hpp11 after chronic administration is crucial. To assess this objective, CAF diet-fed rats were used in this study. These animals are considered as a robust model for human MetS that provides an exceptional tool for screening antihypertensive agents for diet-induced HTN [34].

The results of this study show that the administration of Hpp11 at 55 mg/kg bw for 3 weeks produced a clear decrease in the SBP of CAF-diet rats compared to their counterpart animals administered VH, demonstrating the chronic antihypertensive properties of Hpp11 in this animal model. In this sense, while Hpp11 produced a significant decrease in SBP from the 1st week of administration, the SBP of the CAF+VH group continued to increase as the experiment progressed, reaching final SBP values close to 150 mmHg. These findings demonstrate that Hpp11 produces an antihypertensive preventive effect in CAF diet-fed rats by preventing the continuous increase in SBP triggered by CAF-diet intake. Interestingly, at the 1st week of Hpp11 administration, this hydrolysate was able to reduce SBP as much as Captopril decreased SBP (-19 ± 2 mmHg and -20 ± 3 mmHg, respectively). Moreover, Hpp11 showed a sustained antihypertensive effect from the 1st week of administration until the end of the experiment. Other studies have reported the long-term antihypertensive effects of other protein hydrolysates in SHR [35, 36]. However, the SHR model, which presents an important genetic component, is very different from the CAF-diet fed rats used in this study. As was mentioned previously, the CAF-diet fed rat model is a model of diet-induced HTN. Moreover, HTN in these animals is associated with other cardiometabolic risk factors. In the present study it was observed that after 8 weeks of diet consumption, CAF diet-fed rats presented with obesity, impaired glucose homeostasis and hypertriglyceridemia, in addition to HTN. These findings agree with those reported by Pons [34]. In this sense, since previous studies have reported that some protein hydrolysates derived from other food matrixes, such as fish or soy, affect other cardiometabolic risk factors, such as plasmatic cholesterol [37] or BW [38], the Hpp11 administration

effect was also evaluated on these altered cardiometabolic factors in CAF diet-fed rats. Nevertheless, no changes in BW, glucose homeostasis, plasmatic TG or plasmatic total cholesterol were observed after Hpp11 administration for 3 weeks with respect to the CAF diet-fed rat administered vehicle. Similar results were observed for the CAF rats that were administered Captopril, with no observed improvements in these parameters compared to the CAF group receiving VH.

In addition, in the present study, we researched the mechanisms underlying the long-term antihypertensive effect observed after Hpp11 supplementation in CAF diet-fed rats. One of the mechanisms involved in endothelial damage and the control of BP is oxidative stress. In this sense, in obesity, as a consequence of an alteration in the production of inflammatory cytokines, there is an increase in oxidative stress and in the development of endothelial dysfunction. This endothelial dysfunction is characterized by the increase in the contractibility of the vascular smooth muscle, the scavenging of the vasodilator NO or the stimulation in the production of vasoconstrictors and pro-inflammatory agents [39]. Liver concentration of GSH is a known biomarker for the global oxidative stress status in the body. GSH is considered a key antioxidant that reduces oxidative stress by removing ROS from the system [40]. In this study, Hpp11 clearly produced an increase in the production of hepatic GSH, which indicates an improvement in oxidative stress. In agreement, Liu et al. found that antioxidant effects induced by waste proteins of *Mactra veneriformis* also enhanced the activity of GSH [41]. In addition, other studies revealed antioxidant properties for proteins and peptides that improved oxidative stress by multiple pathways [42].

The renin angiotensin aldosterone system is a key factor in the maintenance of arterial BP, and one of its main components is ACE. Hpp11 was previously reported to inhibit ACE activity after acute administration in SHR [43]. However, we did not find significant changes in the plasmatic ACE activity after long-term Hpp11 administration. Nevertheless, it has also been reported that long-term treatment with ACE-inhibitors can produce an increase in plasmatic ACE concentration in both humans [44] and rats [45]. In agreement, we found that the CAF rats administered the ACE inhibitor Captopril presented an increase in plasmatic ACE activity. Miguel et al. also reported an increase in plasmatic ACE after long-term administration of egg white hydrolysate in SHR [35]. An increase in ACE gene transcription during HTN treatment with ACE inhibitors seems to be responsible for the resulting plasmatic ACE increase [46]. This increase could be an adaptive response to the inhibition of ACE; however, it does not seem to reduce the effectiveness of these drugs.

Furthermore, a potential vasoprotective effect of Hpp11 was studied through the expression of key genes implicated in endothelial function. In this sense, Sanchez et al. reported that the milk casein peptides VPP and IPP reduced BP through ACE inhibition and an increase in eNOS mRNA expression and through the resulting increase in NO availability [36]. However, no changes in the expression of eNOS were obtained after Hpp11 administration, and no changes in the transcription factor KLF-2, which is a potent inducer of eNOS expression, were observed in the rats administered Hpp11. However, Hpp11 produced the upregulation of Sirt-1 mRNA expression. Sirt-1 is a NAD-dependent deacetylase that induces the deacetylation of eNOS and that, consequently, increases NO availability [47]. Therefore, the

involvement of NO in the antihypertensive effect of Hpp11 should not be ruled out.

In addition, the effect of Hpp11 on NOX4 mRNA expression was also evaluated. NOX is the main producer of free radicals in the vasculature [20], and in this study, the mRNA expression of NOX4 was upregulated in the animals administered Hpp11. Nevertheless, unlike NOX isoforms 1 and 2, NOX4 is constitutively active and produces primarily H_2O_2 rather than $^{\bullet}O_2^-$ [38]. In this regard, its pathophysiological role is not clear, although high levels of NOX4 have been associated with the presence of oxidative stress [48]. However, no differences in mRNA expression of NOX4 were found between MetS rats administered the CAF diet or the healthy control group administered the STD diet, with mRNA NOX4 levels excluded as oxidative stress markers in the present study. Additionally, other studies demonstrated that NOX4 might have protective effects. In this sense, Drummond et al. reported that NOX4 exerts vasoprotective effects by increasing NO bioavailability and suppressing cell death pathways in arteries from animals with experimentally induced hypertension, diabetes, or atherosclerosis [49]. In addition, Ray et al. reported that mice with a genetic deletion of NOX4 developed exaggerated contractile dysfunction, hypertrophy, and cardiac dilatation during exposure to chronic overload, indicating that NOX4 enhanced vasodilatation and reduced BP [50]. Therefore, we suggest that the significant upregulation of NOX4 after Hpp11 administration in these animals would result in NOX4-derived vasoprotective effects. The effect of NOX4-derived H_2O_2 as a vasodilator in some vascular beds could be one of the mechanisms responsible for the beneficial effects of Hpp11 [49].

Finally, it was observed that Hpp11 reduced the mRNA expression of ET-1 compared to the CAF+VH group. ET-1 is one of the main

vasoconstrictors in the endothelium that has been reported to be upregulated in obesity and MetS [51]. In addition, apart from the vasoconstrictive action, ET-1 causes fibrosis of the vascular cells and stimulates the production of ROS [52]. The results showed that Hpp11 reduced the expression of ET-1. In this sense, ET-1 release was found to be inhibited after the administration of peptide derived from the bovine β-lactoglobulin ALPMHIR [53], and Fernández-Mussoles reported that lactoferrin hydrolysates inhibited endothelin-converting enzyme, a key enzyme for the production of active ET-1 [54].

5. Conclusions

Hpp11 administration for 3 weeks significantly reduced BP in animals presenting diet-induced HTN. Hpp11 exhibits an antioxidant effect and confers a vasoprotective effect on CAF-diet fed rats, downregulating the aortic mRNA expression of the main endothelial vasoconstrictor, ET-1, and inducing the upregulation of NO-enhancer Sirt-1 mRNA and NOX-4 mRNA expression. Therefore, Hpp11 could be a good candidate for ameliorating diet-induced HTN. Nevertheless, further studies are needed to evaluate its efficiency in humans.

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Conflict of interest

The authors declare no conflict of interest.

References

1. Poulter NR, Prabhakaran D, Caulfield M (2015) Hypertension. Lancet 386:901–812
2. Barceló, M.A; Rodríguez-Poncelas, A.; Saez, M.; Coll-de-Tuero G (2017) The dynamic behaviour of metabolic syndrome and its components in an eight-year population-based cohort from the Mediterranean. 1–18
3. Sampey BP, Vanhoose AM, Winfield HM, et al (2009) Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation : Comparison to high-fat diet. Obesity 19:1109–1117
4. Pons Z, Margalef M, Bravo FI, et al (2015) Acute administration of single oral dose of grape seed polyphenols restores blood pressure in a rat model of metabolic syndrome: role of nitric oxide and prostacyclin. Eur J Nutr
5. Mahjoub S, Roudsari JM (2012) Role of oxidative stress in pathogenesis of metabolic syndrome. Casp J Intern Med 3:386–396
6. Sharma S, Singh R, Rana S (2011) Bioactive peptides: A review. Int J Bioautomation 15:223–250. https://doi.org/10.1093/fqsafe/fyx006
7. Aleixandre A, Miguel M, Muguerza B (2008) Péptidos antihipertensivos derivados de proteínas de leche y huevo. Nutr Hosp 23:313–318
8. Ahhmed AM, Muguruma M (2010) A review of meat protein hydrolysates and hypertension. Meat Sci 86:110–118
9. Ketnawa S, Rawdkuen S (2013) Angiotensin Converting Enzyme Inhibitory Peptides From Aquatic and Their Processing By-Products: a Review. Int J Sci Innov Discov 2:185–199
10. Lasekan A, Abu Bakar F, Hashim D (2013) Potential of chicken by-products as sources of useful biological resources. Waste Manag 33:552–565
11. Mora L, Reig M, Toldrá F (2014) Bioactive peptides generated from meat industry by-products. Food Res Int 65:344–349
12. Mas-Capdevila A, Pons Z, Aleixandre A, et al (2018) Dose-Related Antihypertensive Properties and the Corresponding Mechanisms of a Chicken Foot Hydrolysate in Hypertensive Rats. Nutrients. https://doi.org/10.3390/nu10091295
13. Marques C, Manuela Amorim M, Odila Pereira J, et al (2012) Bioactive Peptides - Are There More Antihypertensive Mechanisms Beyond ACE Inhibition? Curr Pharm Des 18:4706–4713
14. Chakrabarti S, Wu J (2016) Bioactive peptides on endothelial function. Food Sci Hum Wellness 5:1–7
15. Sandoo A, Veldhuijzen van Zanten JJCS, Metsios GS, et al (2010) The endothelium and its role in regulating vascular tone. Open Cardiovasc Med J 4:302–312
16. Förstermann U, Sessa WC (2012) Nitric oxide synthases: Regulation and function. Eur Heart J 33:829–837. https://doi.org/10.1093/eurheartj/ehr304
17. Atkins GB, Jain MK (2007) Role of Krüppel-like transcription factors in endothelial biology. Circ Res 100:1686–1695. https://doi.org/10.1161/01.RES.0000267856.00713.0a
18. Schiffrin EL (2001) Role of endothelin-1 in hypertension and vascular disease. Am J Hypertens 14:83S–89S

19. Pons Z, Margalef M, Bravo FI, et al (2016) Grape seed flavanols decrease blood pressure via Sirt-1 and confer a vasoprotective pattern in rats Grape seed flavanols decrease blood pressure via Sirt-1 and confer a vasoprotective pattern in rats
20. Zarzuelo MJ, López-Sepúlveda R, Sánchez M, et al (2013) SIRT1 inhibits NADPH oxidase activation and protects endothelial function in the rat aorta: Implications for vascular aging. Biochem Pharmacol 85:1288–1296
21. Cai H, Harrison DG (2000) Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 87:840–4
22. Udenigwe CC, Udechukwu MC, Yiridoe C, et al (2016) Antioxidant mechanism of potato protein hydrolysates against in vitro oxidation of reduced glutathione. J Funct Foods 20:195–203. https://doi.org/10.1016/j.jff.2015.11.004
23. Bravo, F.I; Arola, L.; Muguerza B (2017) Procedure for obtaining a hydrolyzate claw chicken leg with antihypertensive activity, and peptides obtained hydrolyzate containing
24. AOAC (1995) Official methods of analysis, 16th ed. Arlington
25. Adler-Nissen J (1979) Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. J Agric Food Chem 27:1256–1262
26. Dahl-Lassen R, van Hecke J, Jørgensen H, et al (2018) High-throughput analysis of amino acids in plant materials by single quadrupole mass spectrometry. Plant Methods 14:1–9. https://doi.org/10.1186/s13007-018-0277-8
27. Guerrero L, Castillo J, Quiñones M, et al (2012) Inhibition of angiotensin-converting enzyme activity by flavonoids: Structure-activity relationship studies. PLoS One 7:1–11
28. Buñag RD, Butterfield J Tail-cuff blood pressure measurement without external preheating in awake rats. Hypertension 4:898–903
29. Kamencic H, Lyon A, Paterson PG, Juurlink BHJ (2000) Monochlorobimane Fluorometric Method to Measure. 37:35–37
30. Martin KA, Mani M V., Mani A (2015) New targets to treat obesity and the metabolic syndrome. Eur J Pharmacol 763:64–74
31. Antonakoudis G, Poulimenos L, Kifnidis K, et al (2007) Blood pressure control and cardiovascular risk reduction. 114–119
32. Chandra A, Neeland IJ, Berry JD, et al (2014) The relationship of body mass and fat distribution with incident hypertension: Observations from the dallas heart study. J Am Coll Cardiol 64:997–1002. https://doi.org/10.1016/j.jacc.2014.05.057
33. Miguel M, Muguerza B, Sánchez E, et al (2005) Changes in arterial blood pressure in hypertensive rats caused by long-term intake of milk fermented by Enterococcus faecalis CECT 5728. Br J Nutr 94:36–43
34. Pons Z, Margalef M, Bravo FI, et al (2018) Chronic administration of grape-seed polyphenols attenuates the development of hypertension and improves other cardiometabolic risk factors associated with the metabolic syndrome in cafeteria diet-fed rats. 200–208
35. Miguel M, Manso MA, Martín- PJ, et al (2007) Angiotensin-converting enzyme activity in plasma and tissues of spontaneously hypertensive rats after the short- and long-term intake of hydrolysed egg white. 555–563
36. Sánchez D, Kassan M, Contreras MDM, et al (2011) Long-term intake of a milk casein hydrolysate attenuates the development of hypertension and involves cardiovascular benefits. Pharmacol Res 63:398–404
37. Wergedahl H, Liaset B, Gudbrandsen OA, et al (2004) Fish protein hydrolysate reduces plasma total cholesterol, increases the proportion of HDL cholesterol, and lowers acyl-CoA:cholesterol acyltransferase activity in liver of Zucker rats.

J Nutr 134:1320–1327

38. Aoyama T, Fukui K, Nakamori T, et al (2000) Effect of soy and milk whey protein isolates and their hydrolysates on weight reduction in genetically obese mice. Biosci Biotechnol Biochem 64:2594–2600
39. Yanai H, Tomono Y, Ito K, et al (2008) The underlying mechanisms for development of hypertension in the metabolic syndrome. Nutr J 7:1–6. https://doi.org/10.1186/1475-2891-7-10
40. Quiñones M, Muguerza B, Miguel M, Aleixandre A (2011) Evidence that nitric oxide mediates the blood pressure lowering effect of a polyphenol-rich cocoa powder in spontaneously hypertensive rats. Pharmacol Res 64:478–481
41. Liu R, Wang L, Zheng W, Wu H (2015) In vivo antioxidant effects of hydrolysate derived from waste proteins of Mactra veneriformis. J Aquat Food Prod Technol 24:143–152
42. Elias RJ, Kellerby SS, Decker EA (2008) Antioxidant activity of proteins and peptides. Crit Rev Food Sci Nutr 48:430–441. https://doi.org/10.1080/10408390701425615
43. Mas-Capdevila, A.; Iglesias-Carres, L.; Pons, Z.; Arola-Arnal, A. ; Bravo, F.I; Muguerza B Relationship between dose and antihypertensive effect for an ACE inhibitor chicken feet hydrolysate. Ann Nutr Metab 73 (2):60
44. Boomsma F, De Bruyn JHB, Derkx FHM, Schalekamp MADH (1981) Opposite effects of captopril on angiotensin I converting enzyme “activity” and “concentration”; relation between enzyme inhibition and long term blood pressure response. Clin Sci 60:491–498
45. Fyhrquist F, Forslund T, Tikkanen I, Gronhagen-riska C (1980) Induction of angiotensin I-converting enzyme in rat lung with captopril (SQ14225). Eur J Pharmacol 67:473–475
46. Costerousse O, Allegrini J, Clozel JP, et al (1998) Angiotensin I-converting enzyme inhibition but not angiotensin II suppression alters angiotensin I-converting enzyme gene expression in vessels and epithelia. J Pharmacol Exp Ther 284:1180–1187
47. Mattagajasingh I, Kim C-S, Naqvi A, et al (2007) SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. Proc Natl Acad Sci 104:14855–14860
48. Konior A, Schramm A, Czesnikiewicz-Guzik M, Guzik TJ (2014) NADPH Oxidases in Vascular Pathology. Antioxid Redox Signal 20:2794–2814. https://doi.org/10.1089/ars.2013.5607
49. Drummond GR, Sobey CG (2014) Endothelial NADPH oxidases: which NOX to target in vascular disease? Trends Endocrinol Metab 25:452–63
50. Ray R, Murdoch CE, Wang M, et al (2011) Endothelial Nox4 NADPH oxidase enhances vasodilatation and reduces blood pressure in vivo. Arterioscler Thromb Vasc Biol 31:1368–1376. https://doi.org/10.1161/ATVBAHA.110.219238
51. Tesauro M, Cardillo C (2011) Obesity, blood vessels and metabolic syndrome. Acta Physiol 203:279–286. https://doi.org/10.1111/j.1748-1716.2011.02290.x
52. Kowalczyk A, Kleniewska P, Kolodziejczyk M, et al (2015) The role of endothelin-1 and endothelin receptor antagonists in inflammatory response and sepsis. Arch Immunol Ther Exp (Warsz) 63:41–52
53. Maes W, Van Camp J, Vermeirssen V, et al (2004) Influence of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg (ALPMHIR) on the release of endothelin-1 by endothelial cells. Regul Pept 118:105–109
54. Fernández-Musoles R, Salom JB, Martínez-Maqueda D, et al (2013) Antihypertensive effects of lactoferrin hydrolyzates: Inhibition of angiotensin- and endothelin-converting enzymes. Food Chem 139:994–1000

TABLES

Table 1. Amino acid profile of chicken foot hydrolysate Hpp11.

Amino acid	(mg/g)
Alanine	20.26 ± 0.53
Arginine	3.36 ± 0.52
Asparagine	n.d.
Aspartic acid	5.22 ± 0.29
Cysteine	0.39 ± 0.05
Glycine	22.04 ± 0.51
Glutamic acid	16.84 ± 0.55
Glutamine	n.d.
Histidine	8.66 ± 0.47
Lysine	4.42 ± 0.64
Methionine	5.42 ± 0.03
Phenylalanine	8.66 ± 0.47
Proline	22.19 ± 2.29
Serine	3.03 ± 0.11
Threonine	2.01 ± 0.29
Tryptophan	0.97 ± 0.19
Tyrosine	9.42 ± 0.18
Valine	9.65 ± 0.29

Results are presented as mean ± standard deviation. N.D, non-identified.

Table 2. Primer list characteristics

Rat primers	Sequence (5'.....3')	Size of amplicon	Efficiency	GenBank accession No.
eNOS Fw	GGATTCTGGCAAGACCGATTAC	159	2.06 (100.67%)	NM_021838.2
eNOS Rv	GGTGAGGACTTGTCCAAACACT			
ET-1 Fw	TGATTCTCTTGCCTCTTCTTG	110	3.08 (136.04%)	NM_012548.2
ET-1 Rv	TATGGAATCTCCTGGCTCTC			
KLF2 Fw	GGACGCACACAGGTGAGAA	186	2.18 (107.85%)	NM_001007684.1
KLF2 Rv	ACATGTGTCGCTTCATGTGC			
Sirt1 Fw	TTGGCACCGATCCTCGAA	217	2.02 (97.49%)	XM_006223877.1
Sirt1 Rv	ACAGAAACCCCAGCTCCA			
NOX4 Fw	GTGTCTGCATGGTGGTGGTA	150	2.24 (110.62%)	NM_053524.1
NOX4 Rv	TCAACAAGCCACCCGAAACA			

Table 3. Body weight and values of triglycerides, cholesterol and glucose in plasma in STD-diet rats and CAF-diet rats supplemented with VH (CAF+VH), 50 mg/kg Captopril (CAF+Captopril) and 55 mg/kg Hpp11 (CAF+Hpp11) at the end of the experiment.

	STD+VH	CAF+VH	CAF+Captopril	CAF+Hpp11
Body weight (g)	431.8 ± 19.64^{a}	517.40 ± 14.66^{b}	501.40 ± 18.30^{b}	537.94 ± 10.34^{b}
Tryglycerides (mmol/L)	0.66 ± 0.11^{a}	1.82 ± 0.08^{b}	2.09 ± 0.37^{b}	2.11 ± 0.20^{b}
Cholesterol (mmol/L)	1.68 ± 0.13^{a}	2.48 ± 0.22^{b}	2.19 ± 0.16^{b}	2.73 ± 0.24^{b}
Glucose (mg/dL)	176.80 ± 7.68^{a}	257.21 ± 15.77^{b}	238.41 ± 11.06^{b}	249.74 ± 14.75^{b}

Data are expressed as the mean of six animals ± SEM. Different letters (a,b) denote significant differences between groups by one-way ANOVA followed by Tukey post hoc analysis according to Levene's test. esults are presented as mean ± standard deviation. N.D, non-identified.

FIGURES

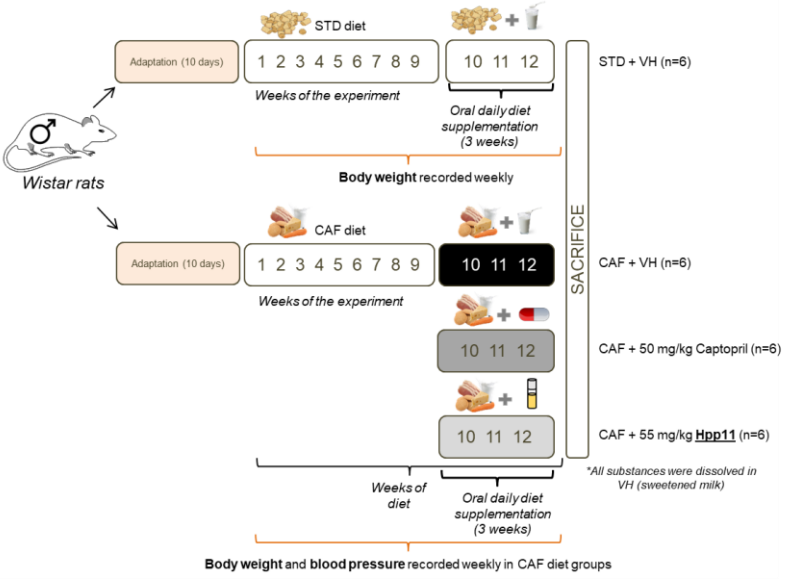

Figure 1. Graphical representation of the experimental design used in this study. STD, standard diet-fed rats; STD+VH, STD-diet rats administered vehicle; CAF, cafeteria diet-fed rats; CAF+VH, CAF-diet rats administered vehicle; CAF+Captopril, CAF-diet rats administered 50 mg/kg Captopril; CAF+Hpp11, CAF-diet rats administered 55 mg/kg Hpp11.

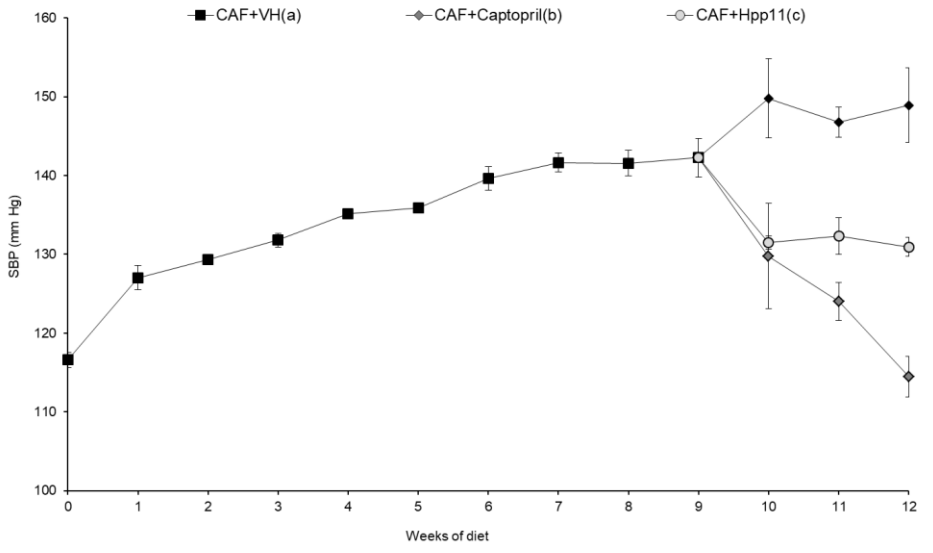

Figure 2. Changes in SBP in rats fed CAF diet for 12 weeks and administered VH (CAF+VH), 50 mg/kg bw of Captopril (CAF+Captopril) or 55 mg/kg bw of Hpp11 (CAF+Hpp11) the last 3 weeks of the experiment. Values are means (n=6 animals per group) with their standard errors. Different letters represent significant differences between groups for the overall effect during the 12 weeks of the experiment (two-way ANOVA; $p<0.05$).

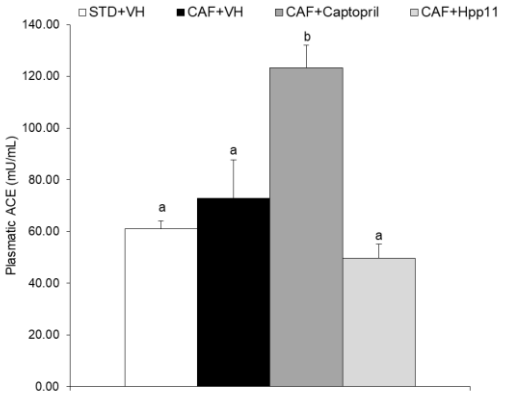

Figure 3. Plasmatic ACE values at the end of the experiment in the STD+VH, CAF+VH, CAF+Captopril (50 mg/kg bw) or CAF+Hpp11 (55 mg/kg bw) groups. Values are means (n=6 animals per group) with their standard errors. Different letters represent significant differences between groups (one-way ANOVA; $p<0.05$).

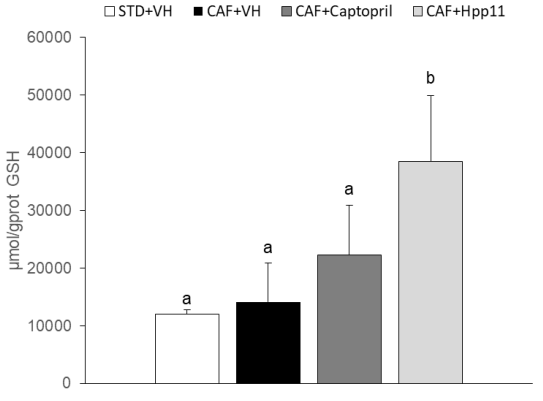

Figure 4. Hepatic GSH production in liver in STD+VH rats and CAF rats supplemented with VH (CAF+VH), 50 mg/kg bw Captopril (CAF+Captopril) or 55 mg/kg Hpp11 (CAF+Hpp11) at the end of the experiment. The data shown are means (n=6 animals per group) with their standard errors. Different letters represent significant differences between groups (one-way ANOVA; $p<0.05$).

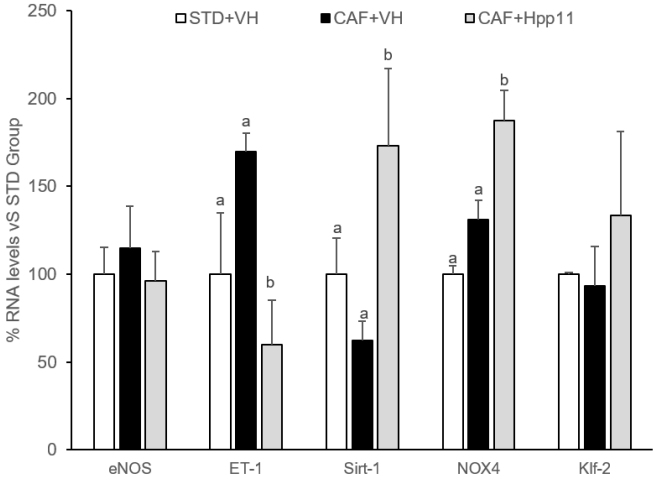

Figure 5. Changes in mRNA expression of genes related to endothelial function in STD+VH, CAF+VH, CAF+Captopril (50 mg/kg bw) and CAF+Hpp11 (55 mg/kg bw) at the end of the experiment. Values are means (n=6 animals per group) with their standard errors. Different letters represent significant differences between groups (one-way ANOVA; $p<0.05$).

CHAPTER 4

Identification of antihypertensive peptides in chicken foot hydrolysate Hpp11

MANUSCRIPT 4

Objective

To isolate and identify angiotensin I-converting enzyme inhibitory (ACEI) peptides from chicken foot hydrolysate Hpp11 and to test their antihypertensive properties.

Novel antihypertensive peptides derived from chicken foot proteins

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Novel antihypertensive peptides derived from chicken foot proteins

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Abbreviations: **ACE,** angiotensin-converting enzyme; **ACEI,** angiotensin-converting enzyme inhibitory; **CVD**, cardiovascular disease; **BP**, blood pressure; **RP-HPLC**, reverse phase high performance liquid chromatography; HPLC-MS, high performance liquid chromatography- mass spectrometry; **SBP**, systolic blood pressure; **DBP**, diastolic blood pressure**; SHR**, spontaneously hypertensive rats

Abstract

Introduction: Chicken foot proteins have recently been demonstrated by our group to be a great source of hydrolysates with antihypertensive properties. The aim of this study was to isolate and identify angiotensin I-converting enzyme inhibitory (ACEI) peptides from chicken foot hydrolysate Hpp11 and to test their antihypertensive properties.

Materials and methods: Peptides were separated into fractions according to their molecular size and hydrophobicity by ultrafiltration and RP-HPLC, respectively. Subsequent peptide identification in the two fractions that presented the highest ACEI activities was carried out by HPLC-MS. Ten of the identified peptides were synthesized and five of them showed ACEI (IC_{50}) values lower than 100 μM. The antihypertensive effects of these ACEI peptides after oral administration was evaluated in spontaneously hypertensive rats.

Results and discussion: The peptides AVFQHNCQE and QVGPLIGRYCG exhibited antihypertensive activity when administered at an oral dose of 10 mg/kg bw. The maximal decrease in systolic blood pressure was recorded 6 h after their administration (25.07 ± 4.21 and 10.94 ± 1.96 mm Hg, respectively).

Conclusion: These results suggest that AVFQHNCQE and QVGPLIGRYCG could be used as functional ingredients with antihypertensive effects, although it would be necessary to perform bioavailability and clinical studies to demonstrate their efficiency in humans.

1. Introduction

In recent years, hypertension has become a public health concern, being one of the independent risk factor for developing cardiovascular diseases (CVD), the leading global cause of death [1]. It is well known that a reduction in blood pressure (BP) is beneficial to prevent CVD. One of the main regulatory systems of the BP is the renin-angiotensin-aldosterone system, being angiotensin I-converting enzyme (ACE, EC 3.4.15.1) the key enzyme in this system. ACE belongs to a family of zinc metallopeptidases and produce the cleavage of the C-terminal dipeptide from angiotensin I to release angiotensin II, a potent vasoconstrictor [2]. The inhibition of ACE is considered important target for treatment of hypertension [3]. However, the administration of synthetic ACE inhibitors, such as Captopril, Lisinopril and Enalapril, has been reported to have adverse side effects [4]. Thus, there has been an increasing interest in development of the natural antihypertensive compounds and ACE inhibitors as alternative for lowering BP [5].

In this sense, bioactive peptides from dietary proteins have reported a wide range of biological activities [6, 7] including antihypertensive effect [8–10]. These peptides are enclosed in the native structure of the proteins and a treatment is needed to release these bioactive sequences [11]. However, other factors such as the possible structural changes performed during the protein digestion and the suitability to be absorbed in the gastrointestinal tract could interfere in the peptides final bioactivity *in vivo* [12]. Therefore, although bioactive peptides presenting *in vitro* ACE inhibitory (ACEI) activity has been extensively reported [13], the evidence for their beneficial antihypertensive effects has to be based on the effect after their administration in animal experiments [14].

Different protein sources have been demonstrated to release ACEI peptides with BP lowering properties after their oral administration [8, 15, 16], including chicken proteins [17–20]. In this sense, chicken foot by-products of the poultry industry, have recently been established by our group as an excellent protein source to obtain hydrolysates with antihypertensive properties [21]. In fact, the BP lowering effect after short and long-term administration of a low dose of the chicken foot hydrolysate Hpp11 have been recently demonstrated in spontaneously hypertensive rats (SHR) and cafeteria-diet fed rats [22, 23]. However, the chicken food bioactive peptides have not been characterized yet. Therefore, the aim of the present study was to isolate and identify the ACEI peptides present in Hpp11. The isolation of the peptides was performed by ultrafiltration followed by two chromatographic steps and their amino acid sequence were identified by mass spectrometry. The antihypertensive effect of peptides that exhibited notable ACEI activity was posteriorly proved in SHR.

2. Materials and methods

2.1. Chemicals and reagents

Chicken feet (*Gallus gallus domesticus*) were provided by a local farm (Granja Gaià, La Riera de Gaià, Spain). Protamex® (EC 3.4.21.62 and 3.4.24.28, 1.5 AU/g from *Bacillus licheniformis and Bacillus amyloliquefaciens*) were kindly provided by Novozymes (Bagsværd, Denmark). ACE (angiotensin converting enzyme, EC 3.4.15.1) was purchased from Sigma-Aldrich (Madrid, Spain), Captopril (PubChem CID: 44093) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and *o*-aminobenzoylglicil-*p*-nitrofenilalanilprolina (o-Abz-Gly-p-Phe(NO_2)-Pro-OH) was provided by Bachem Feinchemikalien (Bubendorf, Switzerland). The synthesized peptides (LSETVV,

LSGPVKF, AVKILP, VRWEPAPGPV, VGKPGARAPMY, QVGPLIGRYCG, LGIHPDWQFV, and AVFQHNCQE, purity grade ≥ 90 %) were purchased from Caslo Laboratory ApS (Caslo, Kongens Lyngby, Denmark). Acetonitrile and trifluoroacetic acid were purchased from Sigma-Aldrich. All other chemical solvents used were of analytical grade.

2.2. Preparation of chicken foot hydrolysate

The chicken foot protein hydrolysate Hpp11 was elaborated using the commercial enzymatic solution Protamex® (Novozymes, Bagsværd, Denmark) (EC 3.4.21.62 and 3.4.24.28, 1.5 AU/g from *Bacillus licheniformis* and *Bacillus amyloliquefaciens*) as previously was described [21]. Briefly, chicken feet were cleaned, crushed, lyophilized, milled and sieved using a 2-mm pore size sieve to obtain a fine chicken foot powder. Chicken foot powder (20 mg/mL, w/v) was resuspended in distilled water and incubated for 90 min in a water bath set at 100 °C at 100 rpm. Subsequently, the Protamex® enzymatic solution was added at a final concentration of 2.67 µg/mL (enzyme/substrate ratio, 0.4 AU/g protein). Hydrolysis was carried out at 50 °C for 2 h at pH 7.0 in a MaxQ Orbital Shaker Thermo Scientific (Thermo Fisher Scientific, Waltham, MA, USA). At the end of the reaction, the enzyme was heat-inactivated (80 °C, 10 min) in a water bath. Then, the hydrolysate was centrifuged at 10,000 ×*g* for 20 min at 4 °C and the supernatant was filtered through a 0.45-µm membrane; finally, the filtrate was collected for analysis.

2.3 Isolation and identification of ACEI peptides from chicken foot hydrolysate

2.3.1. Step I: Protein separation based on protein molecular weight

Hpp11 was subjected to ultrafiltration through two hydrophilic membranes with cut-off values of 3 and 10 kDa cut-off (Centripep, Amicon, Inc., Beverly, MA, USA). The obtained fractions (<3 kDa, 3-10 kDa and >10 kDa) were freeze-dried and kept at -20 ºC until use. The protein concentration and ACEI activity were tested for each fraction at least in duplicate and used for fraction selection.

2.3.2. Step II: Peptide separation based on peptide hydrophobicity

Semipreparative RP-HPLC separations of the <3 kDa fraction were performed on an Agilent Series 1260 HPLC equipped with the Agilent OpenLab CDS ChemStation Edition for LC & LC/MS systems A.01.04 software for data acquisition (Agilent Technologies, Santa Clara, CA, USA). A Europa peptide C18 column (25x1.0 cm i.d., 5 µm particle size, 120 A˚ pore size) (Teknokroma) was used. Mobile phase A was a mixture of water-trifluoroacetic acid (1000:1) and mobile phase B contained a mixture of acetonitrile-trifluoroacetic acid (1000:0.8). Elution was performed in gradient mode as follows: initial conditions 0 % B; 0-40 % B, 0-50 min; 40-45 % B, 50-51 min; 45-90 % B, 51-56 min; and 90-0 % B, 56-57 min. A 10 min post-run was required for column re-equilibration. The flow rate was set at 4 mL/min and analyses were performed at room temperature (RT). Absorbance of the eluent was monitored at 214 nm. The sample concentration was 10 mg/mL (dissolved in water) and the injection volume was 750 µL. Fractions from the HPLC system were freeze-dried and kept at -20 ºC until use. The protein concentration and ACEI activity were also tested for each fraction at least in duplicate and used for fraction selection.

The two fractions showing the most potent ACEI activity, F.3 and F.6, were subsequently subjected to a second semipreparative RP-HPLC separation. The chromatographic conditions were similar but the elution was performed with a linear gradient of solvent B in A, going from 10 % to 20 % B over 40 min at RT or from 20 to 30 % B over 40 min for fractions F.3 and F.6, respectively. A flow rate of 4 mL/min was used in both cases. The sample concentration was 10 mg/mL (dissolved in water) and the injection volume was 750 µL. The ACEI activity and protein concentration were determined in all subfractions at least in duplicate and used for subfraction selection.

2.3.3. Step III: Peptide identification by HPLC-MS

For peptide identification, the most active hydrolysate subfractions (F3.3 and F6.6) obtained in the second RP-HPLC separation were diluted in 0.1 % TFA to a 0.1 mg/mL final concentration. A 10 µL aliquot of each sample was injected into the linear trap quadrupole (LTQ) Orbitrap Velos-PRO (Thermo Scientific). Peptides were loaded onto an EASY-Column (2 cm, ID 100 µm, 5 µm, C18-A1 precolumn) (Thermo Scientific) and then eluted onto an EASY-Column (10 cm, ID 75 µm, 3 µm, C18-A2 analytical column) (Thermo Scientific) at a flow rate of 400 nL/min on a nanoEasy high-performance liquid chromatography (HPLC) instrument (Proxeon) coupled to a nanoelectrospray ion source (Proxeon). The mobile phases used consisted of 0.1 % formic acid/2 % ACN (solvent A) and 0.1 % formic acid in 100 % ACN (solvent B). For the F3.3 fraction, the gradient was 0-45 % B over 80 min, from 45-100 % over 20 min and then 10 min at 100 % B. In contrast, for the F6.6 fraction, the gradient was from 0-35 % B over 40 min, from 35-100 % over 10 min and then 10 min at 100 % B. All mass spectra were acquired in positive-ion mode. Full–scan MS spectra were (m/z 50-2000) were acquired with a target value of 1000000 at a resolution of 30000 at

400 m/z, and the 15 most intense ions were selected for collision-induced dissociation fragmentation in the LTQ with a target value of 10000 and a normalized collision energy of 35 %. Precursor ion charge-state screening and monoisotopic precursor selection were enabled. Singly charged ions and unassigned charge states were rejected. Dynamic exclusion was enabled with a repeat count of 1 and an exclusion duration of 30 s.

Proteome Discoverer 1.4.288 (Thermo) with MASCOT 2.4.1.0 was used to search the IPI_chicken_3.81 fasta database (25992 sequences). The following database search parameters were used: peptide tolerance, 10 ppm; fragment ion tolerance, 0.8 Da; no enzyme and variable modification, methionine oxidation. During peptide identification, probability scores greater than the score fixed by Mascot were considered as significant with a p-value minor than 0.05. The automatic decoy database search function in Protein Discover was enabled to allow estimation of the false discovery rate (FDR).

The identified sequences were subsequently chemically synthesized by Caslo Laboratory ApS.

2.4 ACEI activity

ACEI activity was measured according to Mas-Capdevila et al. [22]. The fluorescence measurements were performed after 30 min in a multiscan microplate fluorimeter (FLUOstar optima, BMG Labtech, Offeuburg, Germany). The excitation and emission wavelengths were 360 and 400 nm, respectively. The software used to process the data was FLUOstar control (version 1.32 R2, BMG Labtech).

A nonlinear fit was performed on the experimental data to calculate the 50 % inhibitory concentration values (IC_{50}) with the PRISM version 4.02 program for Windows (GraphPad Software, Inc. San Diego, CA,

USA). ACEI activity was expressed as a percentage (%) or IC_{50} (µg /µL solution). The determination of the ACEI activity of the samples was performed at least in duplicate. Data are represented as a mean value ± standard deviation (SD).

Protein content was determined by the bicinchoninic acid method using the standard Pierce BCA Protein Assay (ThermoFisher Scientific). The assay was conducted according to the manufacturer's instructions in a microplate format. A calibration standard curve was prepared with seroalbumin bovine. Determination of the protein content was performed at least in duplicate. The results are expressed as the mean ± SD.

2.5 Measurement of blood pressure

Male SHR (17–20 weeks old, weighing 350–400 g) were obtained from Charles River Laboratories España S.A. (Barcelona, Spain). The animals were singly housed in animal quarters at 22 °C with a 12 h light/dark period. They were fed with a standard diet based on chow Panlab A04 (Panlab, Barcelona, Spain) and had access to tap water *ad libitum*.

SHR systolic and diastolic blood pressure (SBP and DBP, respectively) was measured using the tail-cuff method [24] with a noninvasive BP system model (Letica, Hospitalet, Barcelona, Spain). The rats were given a single dose of 10 mg/kg bw of the synthesized peptides, including VGKPGARAPMY, QVGPLIGRYCG, AVFQHNCQE, LSGPVKF or AVKILP, dissolved in tap water in a total volume of 1.5 mL. Peptides were administered to the SHR by gastric incubation through an acute administration between 9 and 10 in the morning.

Positive control rats received 50 mg/kg bw of Captopril, a known ACE-inhibitor, also dissolved in 1.5 mL of tap water, and the negative control rats received 1.5 mL of tap water. SBP was measured in the rats before

peptide administration as well as 2, 4, 6, 8 and 24 h post-administration. Before the measurement, the animals were kept at 38 ºC for 10 min to detect the pulsations of the tail artery. The changes in SBP and DBP were expressed as the differences in these variables before and after the administration of the different peptides. Data are shown as the mean values ± standard error of the mean (SEM) for a minimum of six experiments. Before starting the experiment, all animals were accustomed to the process with a 2-week training period.

The animal protocol followed in this study was approved by Spanish Royal Decree 223/1988 and by the Bioethical Committee of Universitat Rovira i Virgili (reference number 8868 by Generalitat de Catalunya).

Differences between treatments were analyzed by two-way analysis of variance (two-way ANOVA). All the analyses were performed using IBM SPSS Statistics (SPSS, Chicago, IL, US). Outliers were determined using Grubbs' test. Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1 Identification of ACEI peptides

Hpp11 was first subjected to ultrafiltration through 3 kDa and 10 kDa cut-off membranes to separate the peptides according to their molecular size. ACEI activity was determined in the following three obtained fractions: <3 kDa, 3-10 kDa and >10 kDa. The percentages of inhibition obtained were 95.98 ± 0.72 for the <3 kDa fraction, 13.25 ± 2.86 for the 3-10 kDa fraction and 30.50 ± 2.24 for the >10 kDa fraction at a protein concentration of 74.2, 78.3 and 41.7 µg/mL, respectively. The ACEI activity of the <3 kDa fraction was the most active and the expressed as IC_{50} was three and a half times higher than activity found

for Hpp11 hydrolysate (3.6 µg/mL vs. 12.6 µg/mL) (Figure 1). These results indicate that the ACEI activity of Hpp11 is mainly due to small peptides.

The <3 kDa fraction was subjected to semipreparative RP-HPLC to separate the peptides according to their peptide hydrophobicity and eluted as described in Section 2.3.2. The base peak chromatogram for this fraction can be seen in Figure 2A, showing that the hydrolysate was a complex mixture of peptides. Thus, it was divided into 8 fractions (named from F1 to F8), which were collected and lyophilized, and their ACEI activities were measured (Figures 2A and B). Fractions F2 to F6 showed high ACEI activity (≥70 %). The ACEI activity of these selected fractions was also determined and expressed as IC_{50} values (µg of protein/mL) to identify the fraction with the largest amounts of bioactive peptides. Most of the ACEI activity from the <3 kDa fraction occurred in fractions 3 and 6 (F3 and F6), which showed $IC_{50} \leq 2$ µg/mL (1.99 and 1.24 µg/mL, respectively). These two fractions were further purified by a second RP-HPLC step. Figures 3A and B show the RP-HPLC pattern obtained from F3 and F6. Fraction F3 was subdivided into 6 new subfractions (from F3.1 to F3.6) and F6 was subdivided into 8 new subfractions (from F6.1 to F6.8). All fractions showed a specific ACEI activity ≤12 µg/mL (IC_{50}) (Figures 3B, C, D and E). However, fractions F3.3 and F6.6 were remarkably the most active, with IC_{50} values of 0.83 and 0.86 µg/mL, respectively (Figures 3D and E, respectively).

The analysis of these subfractions by mass spectrometry allowed the identification of 772 peptides in fraction F3.3 and 248 peptides in fraction F6.6. Eight of these peptides were subsequently selected and synthesized. Peptide selection was performed according to their high sign intensity, peptide length and amino acid sequence (Table 1). As an

example, Figures 4A and 4B show the MS/MS spectrum of ion m/z 538.2 and 554.3 that matched, corresponding to the sequences AVFQHNCQE and VRWEPAPGPV, respectively. The synthesized peptides showed a wide range of ACEI activity percentages from 0 to 92 % (Table 2). From the eight peptides analyzed, only five peptides showed ACEI activity higher than 50 %. Their IC_{50} values were lower than 100 μM. Two of these peptides stood out for their high ACEI activity, sequences AVKILP and QVGPLIGRYCG, which showed IC_{50} values as low as 7.06 and 11.01 μM, respectively.

3.2 Antihypertensive activity of the synthetic peptides

The antihypertensive effects of the peptides showing the lowest IC_{50} values were evaluated in SHR (Figure 5). Prior to oral administration of the different peptides, SHR presented SBP values of 208 ± 8.0 mmHg and DBP values of 163.3 ± 4.6 mmHg (n= 6 for each treatment). The SBP and DBP of SHR administered water did not significantly change during the 48 h duration of the experiment. As expected, the administration of Captopril (50 mg/kg bw) resulted in an important decrease in the SBP of SHR from 2 h after its administration, with the maximum decrease (-33.67 ± 1.94 mmHg) observed at 6 h post-administration. With respect to DBP, Captopril administration also produced a significant reduction, reaching a maximum decrease 4 h post-administration (-32.97 ± 8.8 mmHg). The antihypertensive effect was maintained to 48 h after Captopril administration.

From the five peptide sequences administered to SHR, including VGKPGARAPMY, QVGPLIGRYCG, AVFQHNCQE, LSGPVKF and AVKILP, the peptides AVFQHNCQE and QVGPLIGRYCG exerted antihypertensive effects when administered at 10 mg/kg bw to SHR (Figures 5A and B). Nevertheless, AVFQHNCQE was the most active peptide. The administration of this peptide produced a significant

decrease in both the SBP and DBP in SHR (-25.07 ± 4.21 mmHg and –17.65 ± 3.24 mm Hg, respectively). The maximum decrease in SBP caused by AVFQHNCQE was observed 6 h post-administration while the maximum decrease in DBP was registered at 2 h. BP returned to initial values 24 h after administration. Regarding QVGPLIGRYCG, it was also observed that this peptide produced a significant decrease in SBP (-10.94 ± 1.96 mm Hg) 6 h post-administration. Nevertheless, no significant reduction in DBP was observed when this peptide was administered. The BP of SHR after VGKPGARAPMY, LSGPVKF and AVKILP administration did not change during the experiment (data not shown).

4. Discussion

It is well-known that the hydrolysis of dietary proteins generates a large number of peptides, including some with potentially bioactive activities [25]. In this sense, the hydrolysis of chicken protein from byproducts has been reported to be a good source for obtaining ACEI and antihypertensive peptides [26]. In a previous study performed by our group, chicken feet dissolved in water were heated at 100 °C and hydrolyzed using Protamex to obtain the hydrolysate Hpp11, which was able to reduce BP when administered to SHR at low doses [21]. The bioactivity of this hydrolysate was assumed to be due to the presence of specific peptide sequences in Hpp11. Thus, the aim of this study was to identify the antihypertensive peptides present in the hydrolysate Hpp11. To assess this objective, Hpp11 was subjected to ultrafiltration using hydrophilic membranes with 3 and 10 kDa cut-off values and ACEI activity of the different obtained fractions was measured. The results showed that the <3 KDa fraction presented the highest ACEI activity. This finding attributes the highest ACEI activity to small

peptides present in this hydrolysate. Similar results were reported by other authors for hydrolysates obtained from other protein sources [27–29]. Subsequently, the <3 kDa fraction was subjected to a two-step RP-HPLC analysis resulting in obtaining of two subfractions (F3.3 and F6.6) selected according to their high ACEI activity. The subsequent analysis of subfractions F3.3 and F6.6 by mass spectrometry allowed the selection of eight peptides (LSETVV, LSGPVKF, AVKILP, VRWEPAPGPV, VGKPGARAPMY, QVGPLIGRYCG, LGIHPDWQFV, AVFQHNCQE), which were subsequently synthesized. As far as we know, none of these peptides had been previously identified to have any bioactivity (search carried out in the BIOPEP database as of October 2018, www.uwm.edu.pl/biochemia/index.php/pl/biopep.

The ACEI activity was lower than 100 μM for five of these identified amino acid sequences, indicating their potential roles as ACE inhibitors. This finding is based on the fact that all the selected peptides comprised between 6-11 amino acids; it has been previously reported that peptides presenting high ACEI activity are short in length and comprising 3 to 12 amino acids [5]. It has also been previously reported that the amino acid composition of the three consecutive C-terminal positions plays an important role in ACE competitive inhibition [30]. However, the amino acid residues required for ACE inhibition at the C-terminal position can be different depending on the peptide size [31]. In general, the presence of hydrophobic aromatic amino acids (Tyr, Phe, or Try), or amino acids with hydrophobic branched side chains (Val, Leu, or Ile) lead to an increase in ACE inhibitory activity [13]. Additionally, many identified ACE inhibitors contain Pro at the C-terminal end [32]. In fact, Pro has a rigid ring structure that can lock the carboxyl group into a favorable conformation, making it able to interact with positively charged

residues in the active site of the enzyme. Regarding the N-terminal position, the presence of branched aliphatic amino acids such as Gly, Val, Leu, and Ile has been reported as good ACE substrates [33]. Thus, the presence of the Pro residue at AVKILP C-terminus, the Tyr residue at the C-terminus and the Val residue at the N-terminus in VGKPGARAPMY, and the Phe residue at the C-terminus and Leu at the N-terminus in LSGPVKF may contribute to their ACEI activity. Regarding the peptide AVFQHNCQE, the presence of glutamic acids in its sequence may contribute to its ACEI effect. Indeed, it is known that glutamic acid may cause a net negative charge, and the interaction of negatively charged peptides with ACE could chelate zinc atoms, which is a component of the ACE active center [34]. However, the peptide sequence for QVGPLIGRYCG, one of the most potent ACE inhibitors identified in the Hpp11, did not have any particular structural features that could be responsible for its activity. Nevertheless, the presence of Tyr at the third position before the final position at the C-terminus or its conformation in solution could highly favor its ability to inhibit ACE [35]. In this sense, peptide conformation, that is the structure adopted in the specific environment of the binding site, has been suggested to enhance the inhibitory ability of long-chain peptides [36, 37].

Additionally, two of the peptides, AVKILP and QVGPLIGRYCG, stood out for their high ACEI activities, showing IC_{50} values as low as 7.06 and 11.01 μM, respectively. Although the ACEI activity of the drug Captopril is higher than that presented by these peptides, the interest in dietary bioactive compounds is increasing significantly because they present high tissue affinity, specificity and efficiency in promoting these health effects [38]. Moreover, the ACEI values of chicken foot-derived peptides are similar than those observed for known antihypertensive peptides, such as the fermented milk-derived

peptides IPP, VPP and LHLPLP, which showed similar IC_{50} values (5, 9 and 5.5 μM, respectively) [39, 40]. Other byproduct chicken-derived peptides have been reported to exert high ACEI activities, but their activities ranged from 34 to 254 μM [41, 42].

Many peptides that present ACEI activities *in vitro* do not exhibit antihypertensive properties. In fact, after their oral administration, the peptides may be susceptible to degradation by gastrointestinal enzymes and by brush border, blood serum and/or intracellular peptidases before being transferred into the bloodstream [13]. Additionally, even though some peptides are resistant to digestive enzymes, large peptides (>6 amino acid residues) may not be absorbed into small intestinal epithelial cells [12]. Therefore, testing the *in vivo* antihypertensive effects of the peptides is necessary for validation. To assess their effects *in vivo*, the ACEI peptides identified in Hpp11 were administered to SHR, an experimental animal model that best mimics essential hypertension in humans [43].

The VGKPGARAPMY, LSGPVKF and AVKILP peptides sequences did not exhibit BP-lowering effects. Interestingly, the AVKILP peptide, which showed the most potent ACEI activity, did not present antihypertensive effects when administered to SHR, suggesting that modifications incurred during gastrointestinal digestion could inactivate this peptide. In contrast, the AVFQHNCQE and QVGPLIGRYCG peptides decreased BP after their oral administration (-25.07 and -10.94 mm Hg of SBP at 6 h post-administration, respectively), with AVFQHNCQE being the most active peptide. Interestingly, this peptide was also able to reduce DBP, suggesting its huge potential as an antihypertensive agent. Our findings agree with those demonstrating the potential antihypertensive properties of bioactive peptides from chicken by-products including chicken bone

[17]. Furthermore, the ACEI peptides IKW, LKP, and IVGRPRHQG, which were isolated from chicken muscle, showed similar antihypertensive effects as AVFQHNCQE but needed to be administered at a higher dose of 60 mg/kg bw [44].

5. Conclusions

Novel ACEI peptides have been identified in the chicken foot hydrolysate Hpp11. Moreover, the antihypertensive properties of the QVGPLIGRYCG and AVFQHNCQE peptides have been demonstrated in this study for the first time. The potential of these peptides for use in functional foods to mitigate hypertension appears to merit further clinical studies in humans.

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Conflict of interest

The authors declare no conflict of interest.

References

[1] Celermajer, D.S., Chow, C.K., Marijon, E., Anstey, N.M., et al., Cardiovascular disease in the developing world: Prevalences, patterns, and the potential of early disease detection. *J. Am. Coll. Cardiol.* **2012**, *60*, 1207–1216.

[2] Atlas, S. a, The renin-angiotensin aldosterone system: pathophysiological role and pharmacologic inhibition. *J. Manag. Care Pharm.* **2007**, *13*, 9–20.

[3] Foëx, P., Sear, J.W., Hypertension: Pathophysiology and treatment. *Contin. Educ. Anaesthesia, Crit. Care Pain* **2004**, *4*, 71–75.

[4] Alderman, C.P., Angiotensin-converting enzyme inhibitors. 1994, *30*, 55–61.

[5] Margalef, M., Bravo, F.I., Arola-Arnal, A., Muguerza, B., Natural angiotensin converting enzyme (ACE) inhibitors with antihypetensive properties, in: Andrade, P.., Valentao, P., Pereira, D.M. (Eds.), *Natural Products Targeting Clinically Relevant Enzymes*, First, Wiley-VCH Verlag GmbH & Co. , Weinheim **2017**, pp. 45–67.

[6] Yoshikawa, M., Bioactive peptides derived from natural proteins with respect to diversity of their receptors and physiological effects. *Peptides* **2015**, *72*, 208–225.

[7] Korhonen, H., Pihlanto, A., Bioactive peptides: Production and functionality. *Int. Dairy J.* **2006**, *16*, 945–960.

[8] Yamamoto, N., Antihypertensive peptides derived from food proteins. *Biopolym. - Pept. Sci. Sect.* **1997**, *43*, 129–134.

[9] Hong, F., Ming, L., Yi, S., Zhanxia, L., et al., The antihypertensive effect of peptides: A novel alternative to drugs? *Peptides* **2008**, *29*, 1062–1071.

[10] Hernández-Ledesma, B., Del Mar Contreras, M., Recio, I., Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Adv. Colloid Interface Sci.* **2011**, *165*, 23–35.

[11] Aluko, R.E., Antihypertensive Peptides from Food Proteins. *Annu. Rev. Food Sci. Technol.* **2015**, *6*, 235–262.

[12] Vermeirssen, V., Camp, J. Van, Verstraete, W., Bioavailability of angiotensin I converting enzyme inhibitory peptides. *Br. J. Nutr.* **2004**, *92*, 357.

[13] Meisel, H., Walsh, D.., Murray, B., FitzGerald, R.., ACE inhibitory peptides, in: Mine, Y., Shahidi, F. (Eds.), *Nutraceutical Proteins and Peptides in Health and Disease*, CRC/Taylor and Francis, Boca raton **2006**, pp. 269–316.

[14] Martínez-Maqueda, D., Miralles, B., Recio, I., Hernández-Ledesma, B., Antihypertensive peptides from food proteins: a review. *Food Funct.* **2012**, *3*, 350.

[15] Hernández-ledesma, B., Contreras, M., Recio, I., Antihypertensive peptides : Production , bioavailability and incorporation into foods. *Adv. Colloid Interface Sci.* **2011**, *165*, 23–35.

[16] Iwaniak, A., Minkiewicz, P., Darewicz, M., Food-originating ACE inhibitors, including antihypertensive peptides, as preventive food components in blood pressure reduction. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 114–134.

[17] Nakade, K., Kamishima, R., Inoue, Y., Ahhmed, A., et al., Identification of an antihypertensive peptide derived from chicken bone extract. *Anim. Sci. J.* **2008**, *79*, 710–715.

[18] Onuh, J.O., Girgih, A.T., Malomo, S.A., Aluko, R.E., et al., Kinetics of in vitro renin and angiotensin converting enzyme inhibition by chicken skin protein hydrolysates and their blood pressure lowering effects in spontaneously hypertensive rats. *J. Funct. Foods* **2015**, *14*, 133–143.

[19] Yoshii, H., Tachi, N., Ohba, R., Sakamura, O., et al., Antihypertensive effect of ACE inhibitory oligopeptides from chicken egg yolks. *Comp. Biochem. Physiol. - C Pharmacol. Toxicol. Endocrinol.* **2001**, *128*, 27–33.

[20] Iwai, K., Saiga-Egusa, A., Hayakawa, T., Shimizu, M., et al., An Angiotensin I-converting Enzyme (ACE)-inhibitory Peptide Derived from Chicken Collagen Hydrolysate Lowers Blood Pressure in Spontaneously Hypertensive Rats. *J. JAPANESE Soc. FOOD Sci. Technol. SHOKUHIN KAGAKU KOGAKU KAISHI* **2008**, *55*, 602–605.

[21] Bravo, F.I; Arola, L.; Muguerza, B., Procedure for obtaining a hydrolyzate claw chicken leg with antihypertensive activity, and peptides obtained hydrolyzate containing, **2017**.

[22] Mas-Capdevila, A., Pons, Z., Aleixandre, A., Bravo, F.I., et al., Dose-Related Antihypertensive Properties and the Corresponding Mechanisms of a Chicken Foot Hydrolysate in Hypertensive Rats. *Nutrients* **2018**.

[23] Mas-Capdevila, A.; Iglesias-Carres, L.; Arola-Arnal, A.; Muguerza, B.; Bravo, F.., Antihypertensive effect of chicken-feet hydrolysate (Hpp11) long-term administration in an animal model of metabolic syndrome. X Seminario sobre Alimentación y Estilos de Vida Saludables 1r curso de actualización sobre NUTRIGENÓMICA Y NUTRICIÓN PERSONALIZ, in: NUTRICIÓN, X.S. sobre A. y E. de V.S. 1r curso de actualización sobre N.Y. (Ed.), *Antihypertensive Effect of Chicken-Feet Hydrolysate (Hpp11) Long-Term Administration in an Animal Model of Metabolic Syndrome*, **2018**, pp. 43–44.

[24] Buñag, R.D., Validation in awake rats of a tail-cuff method for measuring systolic pressure. *J. Appl. Physiol.* **1973**, *34*, 279–282.

[25] Udenigwe, C.C., Howard, A., Meat proteome as source of functional biopeptides. *Food Res. Int.* **2013**, *54*, 1021–1032.

[26] Lasekan, A., Abu Bakar, F., Hashim, D., Potential of chicken by-products as sources of useful biological resources. *Waste Manag.* **2013**, *33*, 552–565.

[27] Quirós, A., Ramos, M., Muguerza, B., Delgado, M.A., et al., Identification of novel antihypertensive peptides in milk fermented with Enterococcus faecalis. *Int. Dairy J.* **2007**, *17*, 33–41.

[28] Saiga, A., Iwai, K., Hayakawa, T., Takahata, Y., et al., Angiotensin I-converting enzyme-inhibitory peptides obtained from chicken collagen hydrolysate. *J. Agric. Food Chem.* **2008**, *56*, 9586–9591.

[29] Malomo, S.A., Onuh, J.O., Girgih, A.T., Aluko, R.E., Structural and antihypertensive properties of enzymatic hemp seed protein hydrolysates. *Nutrients* **2015**, *7*, 7616–7632.

[30] Cushman, D., Ondetti, M., Design of angiotensin converting enzyme inhibitors. *Nat. Med.* **1999**, *5*, 1110–1112.

[31] Wu, J., Aluko, R.E., Nakai, S., Structural requirements of angiotensin I-converting enzyme inhibitory peptides: Quantitative structure-activity relationship modeling of peptides containing 4-10 amino acid residues. *QSAR Comb. Sci.* **2006**, *25*, 873–880.

[32] Cushman, D.W., Cheung, H.S., Sabo, E.F., Ondetti, A., Design of Potent Competitive Inhibitors of Angiotensin-Converting Enzyme. Carboxyalkanoyl and Mercaptoalkanoyl Amino Acids. *Biochemistry* **1977**, *16*, 5484–5491.

[33] Li, G.H., Le, G.W., Shi, Y.H., Shrestha, S., Nutrition research., Elsevier Inc,

2004.

[34] Wei, L., Clauser, E., Alhenc-Gelas, F., Corvol, P., The two homologous domains of human angiotensin I-converting enzyme interact differently with competitive inhibitors. *J. Biol. Chem.* **1992**, *267*, 13398–13405.

[35] Cushman, D.W., Cheung, H.S., Sabo, E.F., Ondetti, M.A., Design of potent competitive inhibitors of angiotensin-converting enzyme. Carboxyalkanoyl and mercaptoalkanoyl amino acids. *Biochemistry* **1977**, *16*, 5484–91.

[36] Cheung, H.S., Wang, F.L., Ondetti, M.A., Sabo, E.F., et al., Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Importance of the COOH-terminal dipeptide sequence. *J. Biol. Chem.* **1980**, *255*, 401–407.

[37] FitGerald, R.., Murray, B.., Walsh, D.J., Hypotensive Peptides from Milk Proteins. *J. Nutr.* **2004**, *134*, 980S–988S.

[38] Daliri, E., Oh, D., Lee, B., Bioactive Peptides. *Foods* **2017**, *6*, 32.

[39] Nakamura, Y., Studies on Anti-Hypertensive Peptides in Milk Fermented with Lactobacillus helveticus. *Biosci. Microflora* **2004**, *23*, 131–138.

[40] Quirós, A., Ramos, M., Muguerza, B., Delgado, M. a., et al., Identification of novel antihypertensive peptides in milk fermented with Enterococcus faecalis. *Int. Dairy J.* **2007**, *17*, 33–41.

[41] Gu, R.Z., Liu, W.Y., Lin, F., Jin, Z.T., et al., Antioxidant and angiotensin I-converting enzyme inhibitory properties of oligopeptides derived from black-bone silky fowl (Gallus gallus domesticus Brisson) muscle. *Food Res. Int.* **2012**, *49*, 326–333.

[42] Nakade, K., Kamishima, R., Inoue, Y., Ahhmed, A., et al., Identification of an antihypertensive peptide derived from chicken bone extract. *Anim. Sci. J.* **2008**, *79*, 710–715.

[43] Hasenfuss, G., Animal models of human cardiovascular disease, heart failure and hypertrophy. *Cardiovasc. Res.* **1998**, *39*, 60–76.

[44] Fujita, H., Yokoyama, K., Yoshikawa, M., Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins. *J. Food Sci.* **2000**, *65*, 564–569.

TABLES

Table 1. Identification of the peptides contained in the obtained RP-HPLC subfractions showing the highest angiotensin converting enzyme inhibitory activities.

Fraction	Sequence[a]	Theoretical M.W.	MH+ (Da)	m/z[c] (Da)	Charge
F3.3	VGKPGARAPmY[b]	1146.4	1162.59	581.80	2
F3.3	QVGPLIGRYCG	1162.4	1162.61	581.80	2
F3.3	LGIHPDWQFV	1211.4	1211.62	404.54	3
F3.3	AVFQHNCQE	1075.2	1075.47	538.24	2
F6.6	LSETVV	646.7	647.36	324.18	2
F6.6	LSGPVKF	746.9	747.44	374.22	2
F6.6	AVKILP	639.8	640.44	320.72	2
F6.6	VRWEPAPGPV	1107.3	1107.59	554.30	2

[a] Amino acid residues are designated using their one letter codes.

[b] m= Oxidation of methionine

[c] m/z=mass-to-charge

Table 2. The angiotensin-converting enzyme inhibitory activities of the synthetic peptides expressed as percentages and IC_{50} values (concentration of peptide needed to inhibit 50 % of the original ACE activity).

Fraction	Sequence	Peptide concentration (μM)	ACEI activity	
			%*	IC_{50} (μM)
F3.3	VGKPGARAPmY	174.5	75.8 ± 0.06	29.71
F3.3	QVGPLIGRYCG	161.4	87.2 ± 0.33	11.01
F3.3	LGIHPDWQFV	137.6	0.0 ± 0.0	>137.6
F3.3	AVFQHNCQE	142.8	90.4 ± 0.02	44.75
F6.6	LSETVV	128.9	12.9 ± 0.71	> 515.4
F6.6	LSGPVKF	122.7	58.4 ± 4.27	80.91
F6.6	AVKILP	101.4	91.8 ± 0.03	7.06
F6.6	VRWEPAPGPV	150.5	21.7 ± 2.30	>150

*Percentage of ACEI activity showed at the indicated protein concentration

FIGURES

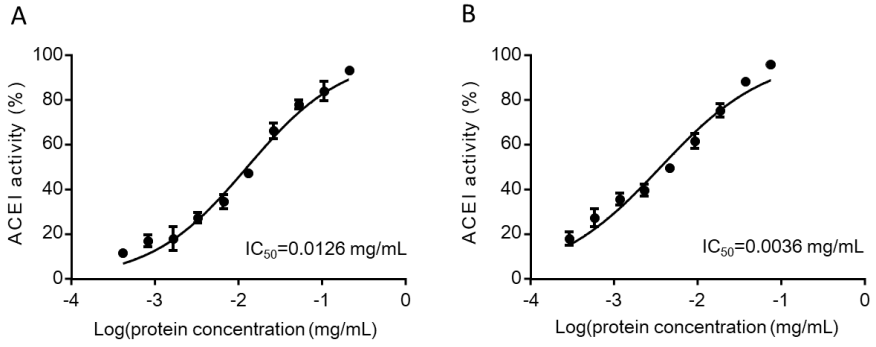

Figure 1. Determination of the angiotensin-converting enzyme inhibitory activity expressed as the IC_{50} for Hpp11 hydrolysate (A) and the Hpp11 hydrolysate <3 kDa fraction (B). The IC_{50} values were determined by curve fitting with a nonlinear regression analysis. The experimental data in each graphic correspond to two different assays in duplicate.

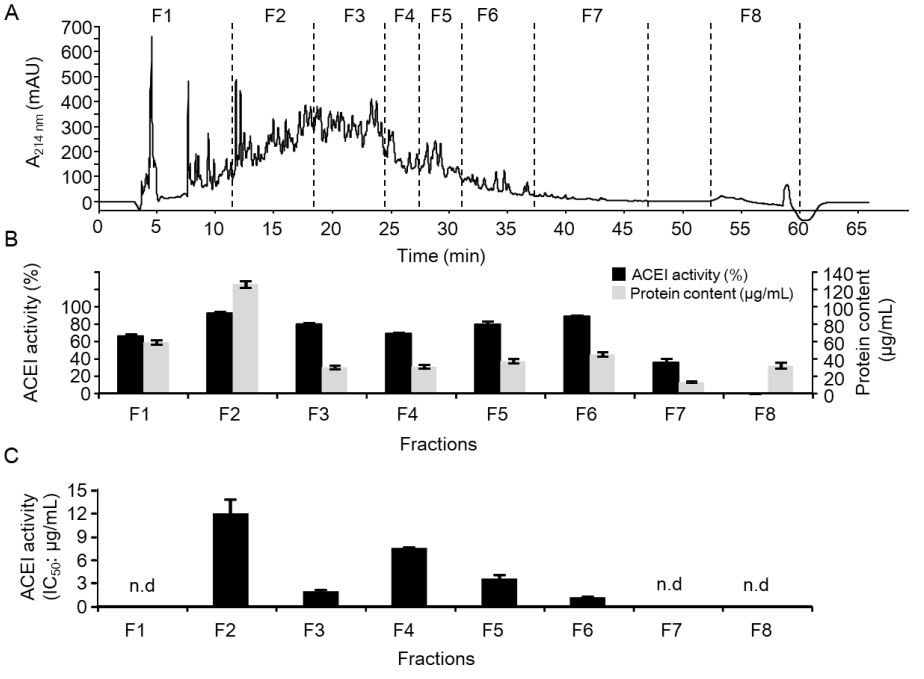

Figure 2. Fractionation by RP-HPLC at a semipreparative scale for the <3 kDa fraction obtained from Hpp11 (A). The collected fractions were termed with F followed by a number (F1–F8). The angiotensin-converting enzyme inhibitory activity (ACEI) expressed as the percentage of inhibition (B) and IC_{50} (μg/mL) (C) of the collected fractions from the semipreparative RP-HPLC system. The data are expressed as the mean ± standard deviation for a minimum of two measurements. The protein contents of the fractions were estimated using the bicinchoninic acid assay. N.d; Non-determined.

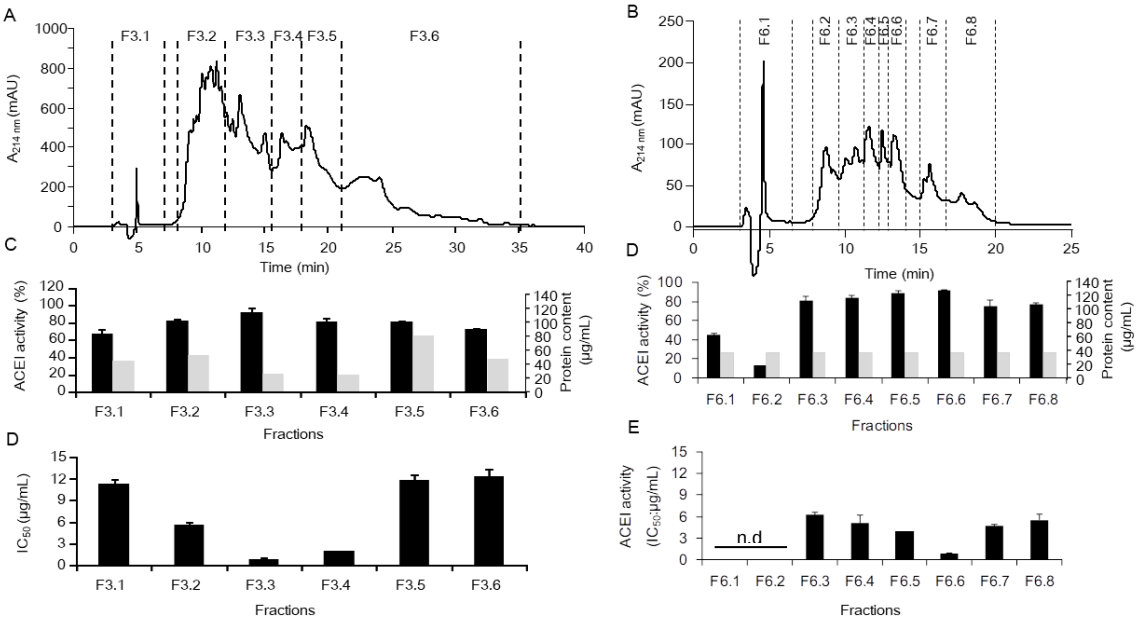

Figure 3. Fractionation by RP-HPLC at a semipreparative scale for fractions F3 (A) and F6 (B) obtained from the first RP-HPLC separation. The collected subfractions are termed with the name of the original fraction followed by a point and a number. The angiotensin-converting enzyme inhibitory activity (ACEI) expressed as the percentage of inhibition (B, C) and IC_{50} (µg/mL) (D, E) of the subfractions from the second separation of F3 and F6, respectively. The data are expressed as the mean ± standard deviation for a minimum of two measurements. The protein contents of the fractions were estimated using the bicinchoninic acid assay. N.d; Non-determined.

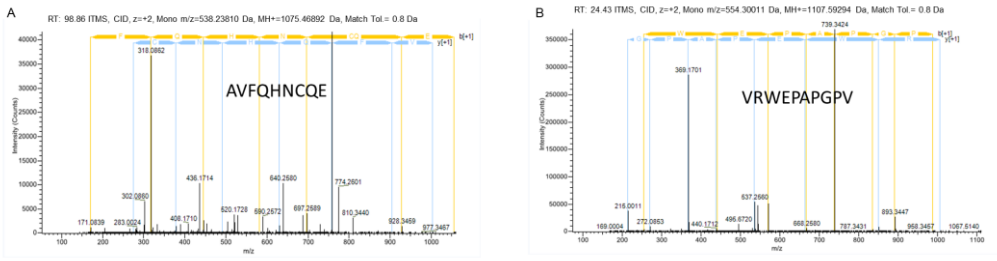

Figure 4. MS/MS spectrum of the doubled charged ions m/z 538.2 (A) and 554.3 (B). Following sequence interpretation and database searching, the peptides were identified as AVFQHNCQE and VRWEPAPGPV, respectively. The MS/MS spectra were acquired with linear trap quadrupole-Orbitrap mass spectrometry. The sequences of these peptides are displayed with the fragment ions observed in the spectra.

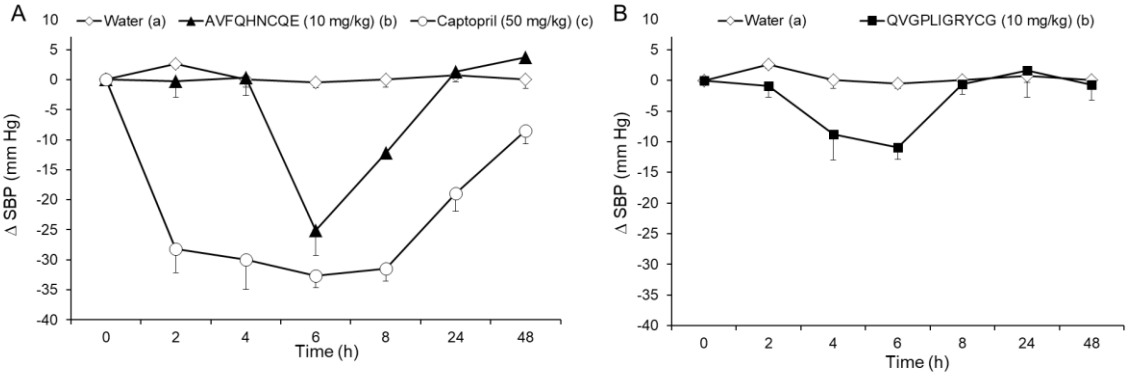

Figure 5. Decrease in the systolic blood pressure (SBP) in spontaneously hypertensive rats after the administration of water, Captopril (50 mg/kg bw) or AVFQHNCQE (10 mg/kg bw) (A) or after the administration of water and QVGPLIGRYCG (10 mg/kg bw) (B). The data are expressed as the mean ± SEM. All of the experimental groups include a minimum of 6 animals. Different letters represent significant differences ($p < 0.05$). p was estimated by two-way ANOVA.

CHAPTER 5

To study the bioavailability and the mechanism of action of AVFQHNCQE and establish the molecular mechanisms involved in its blood-pressure lowering effect

MANUSCRIPT 5

Objective

To evaluate the involvement of vasodilators nitric oxide (NO) and prostacyclin (PGI_2) in the peptide antihypertensive effect in spontaneously hypertensive rats. Additionally, to evaluate the molecular mechanisms under AVFQHNCQE antihypertensive effect.

Evidence that nitric oxide is involved in the blood pressure lowering effect of AVFQHNCQE in spontaneously hypertensive rats

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Abstract

AVFQHNCQE is an antihypertensive nonapeptide obtained from chicken foot protein. The present study aims to investigate the mechanisms involved in the blood pressure (BP)-lowering effect of the peptide. Male (17-20 weeks old) spontaneously hypertensive rats (SHR) were used in this study. Rats were divided into two groups and orally administered water or 10 mg/kg body weight (bw) AVFQHNCQE. One hour post-administration, animals of both groups were intraperitoneally treated with 1 mL of saline or with 1 mL of saline containing 30 mg/kg bw N_{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) synthesis, or with 1 ml of saline containing 5 mg/kg bw indomethacin, inhibitor of prostacyclin synthesis (n= 6 per group). Systolic BP (SBP) was recorded before and 6 h after oral administration. In an additional experiment, SHR were administered water or 10 mg/kg bw AVFQHNCQE (n=6 per group) and sacrificed 6 h post-administration. The aortic expression levels of different genes implicated in endothelial function, activity of plasma angiotensin-converting enzyme (ACE) and level of liver reduced glutathione (GSH) were evaluated in both groups. Moreover, the relaxation caused by AVFQHNCQE in isolated aortic rings from Sprague-Dawley rats was evaluated. The BP-lowering effect of the peptide was not changed after indomethacin administration but was completely abolished by L-NAME, demonstrating that this antihypertensive activity is mediated by changes in endothelium-derived NO availability. In addition, AVFQHNCQE administration downregulated aortic gene expression of the vasoconstrictor factor endothelin-1 and the endothelial major free radical producer NADPH. Moreover, while no changes in plasma ACE activity were observed after its administration, liver GSH levels were higher in the peptide-

treated group than in the water group, demonstrating that AVFQHNCQE also presents antioxidant properties.

Keywords: bioactive peptide, endothelial dysfunction, blood pressure, bioactivity, nitric oxide.

Abbreviations: **ACE,** angiotensin-converting enzyme; **HTN**, hypertension; **BP**, blood pressure; **SD**, sprague dawley; **SHR**, spontaneously hypertensive rats; **SBP**, systolic blood pressure; **L-NAME**, N_{ω}-nitro-L-arginine methyl ester; **GSH,** reduced gluthathione; **ROS**, reactive oxygen species.

1. Introduction

Hypertension (HTN) is a chronic elevation of systemic arterial blood pressure (BP) above certain threshold values. However, the elevation in BP is only a manifestation of a progressive disease representing an important health problem [1]. HTN is considered the most preventable risk factor for cardiovascular disease (CVD), and successful HTN prevention and treatment are key to reducing the risk of CVD [2]. Thus, the current method to treat HTN is the use of long-term drug therapy; however, the use of these drugs can result in different side effects in some patients, leading to reduced patience compliance with the treatment, increased health care costs and preventable fatalities [3,4]. In this context, new strategies for treating HTN based on natural products could greatly benefit hypertensive patients.

Some studies have demonstrated the antihypertensive properties of dietary-derived peptides [5–7]. These bioactive peptides are potential modulators of various regulatory processes that control BP. The renin angiotensin aldosterone system (RAAS) plays an important role in regulating arterial pressure [13]. In RAAS, angiotensin-converting enzyme (ACE) is a key enzyme in the control of BP and converts angiotensin I to the potent vasoconstrictor angiotensin II [8]. Thus, the inhibition of this enzyme has become an important target for drugs combating HTN. In fact, the inhibition of this enzyme is the main mechanism implied in the antihypertensive effect of dietary peptides [9–11]. In addition, endothelial function, which maintains normal vascular tone and blood fluidity to maintain normotensive BP values, has been demonstrated to be the target for some antihypertensive peptides [12–14]. In HTN, endothelial function is impaired, presenting decreased presence of vasodilator factors, such as nitric oxide (NO) or

prostacyclin (PGI_2), and/or increased presence of endothelium-derived contracting factors, such as endothelin 1 (ET-1) [15]. In this sense, some peptides have been demonstrated to present NO-mediated antihypertensive effects [13,16]. NO is synthesized by the enzyme endothelial NO synthase (eNOS), which uses arginine as a substrate to produce NO. L-arginase (Arg-1), the enzyme that degrades arginine, has been demonstrated to play a crucial role in NO production and in the development of vascular disease [17]. In contrast, Krüpple-like factor 2 (KLF-2) has been demonstrated to be an eNOS promoter [18]. KLF-2 can also inhibit the expression of other important genes involved in regulating vessel tone, such as ET-1 [19]. KLF-2 activation is regulated by Sirtuin-1 (Sirt-1), which is also well known to activate eNOS by deacetylation and thereby increases the production of NO and promotes endothelial-dependent vasodilatation [20]. In addition, Sirt-1 promotes the inhibition of the activity of NADPH oxidase (NOX) [21], the major free radical producer in the endothelium, which is known to be overexpressed in spontaneously hypertensive rats (SHR) linking to the presence of endothelial dysfunction in this animal model [22]. Similarly, reactive oxygen species (ROS) have several effects on vascular and endothelial function, such as the inactivation of the vasodilator NO by O^{2-} [23], contributing to the development of endothelial dysfunction [15]. Some studies have demonstrated that antihypertensive peptides can also present antioxidant activity by enhancing one of the main endogenous antioxidant system, reduced glutathione (GSH) [24,25].

In a previous study, the peptide sequence AVFQHNCQE was identified in the antihypertensive chicken foot hydrolysate Hpp11 [26]. This peptide presented *in vitro* ACE inhibitory (ACEI) activity and exhibited antihypertensive activity in SHR at a dose of 10 mg/kg body weight

(bw). Nevertheless, the underlying mechanisms involved in the antihypertensive effect of AVFQHNCQE are still unknown. Thus, the aim of this study was to evaluate the involvement of endothelial-relaxing factors as possible antihypertensive mechanisms of AVFQHNCQE. We used SHR alternatively treated with N_{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthesis, or with indomethacin, an inhibitor of prostacyclin synthesis. Furthermore, in an additional experiment, the concentration of liver GSH, activity of plasma ACE and aortic expression levels of different genes implicated in endothelial dysfunction were evaluated in AVFQHNCQE-treated SHR.

2. Materials and methods

2.1. Reagents

L-NAME, indomethacin, ACE (peptidyl-dipeptidase A, EC 3.4.15.1), N-hippuryl-His-Leu (Hip-His-Leu), monochlorobimane, glutathione-S transferase from horse liver, acetylcholine and methoxamine hydrochloride were purchased from Sigma-Aldrich (Madrid, Spain). The peptide AVFQHNCQE was synthetized by CASLO Aps (Kongens Lyngby, Denmark), and its purity of 98.94% was verified by HPLC–MS. Heparin was purchased from DeltaLab (Barcelona, Spain). All other chemical solvents used were of analytical grade.

2.2. Experimental procedure in animals

Male SHR (17–20 weeks old) weighing 298 ± 2 g were used. All animals were obtained from Charles River Laboratories (Barcelona, Spain). The animals were maintained at a temperature of 23 °C with 12 h light/dark cycles and consumed tap water and a standard diet (A04 Panlab, Barcelona, Spain) *ad libitum* during the experiments. Animals were

administered distilled water or 10 mg/kg bw AVFQHNCQE dissolved in tap water by oral gavage between 8 and 9 am. The total volume orally administered to the rats was 1 mL in all cases. One hour after the oral administration, animals in each group (water or peptide) were intraperitoneally administered 1 mL saline solution (water + saline or AVFQHNCQE + saline groups, respectively). The remaining rats were divided into four groups, two groups that were intraperitoneally administered 30 mg/kg bw L-NAME (water + 30 mg/kg L-NAME or AVFQHNCQE + 30 mg/kg L-NAME) and two groups that were administered 5 mg/kg bw indomethacin (water + 5 mg/kg indomethacin or AVFQHNCQE + 5 mg/kg indomethacin groups) (n=6 for group). L-NAME and indomethacin were dissolved in saline solution, and the volume injected into rats was 1 mL in all treatment groups. Systolic blood pressure (SBP) was recorded in the rats by the tail cuff method [27] before and 6 h after water or peptide administration. To guarantee the reliability of the measurements, we established a training period of 10 days before the actual trial time, and during this period, the rats became accustomed to the procedure. Moreover, to minimize stress-induced variations in BP, all measurements were taken by the same person in the same peaceful environment. Nevertheless, the researcher assigned to carry out the measurements did not know the exact treatment of each animal.

Additional experiment was performed using 17–20-week-old male SHR weighing 302 ± 4 g. Housing and diet conditions were the same as those described in the previous experiment. Animals were administered 1 mL of tap water (n= 6) or 10 mg/kg bw AVFQHNCQE dissolved in 1 mL of tap water (n= 6) by oral gavage and sacrificed by live decapitation 6 h post-administration. Total blood was collected in heparin tubes. Plasma was obtained by blood centrifugation (1,500 x g,

15 min, 4 °C), and aorta and liver were excised and immediately frozen in liquid nitrogen. Both plasma and tissues were stored at −80 °C until further use.

Figure 1 shows a graphical representation of both experimental designs used in this study.

2.3 Determination of plasmatic ACE activity

ACE activity was measured in the plasma following the method reported by Mas-Capdevila et al. [28]. Commercial ACE at different concentrations was used to obtain a calibration curve. Plasma ACE activity was expressed (mU ACE/mL) as the mean ± standard error of the mean (SEM) of plasma for at least three replicates.

2.4 Reduced gluthatione assay

The GSH assay was performed in the liver following the monochlorobimane fluorometric method [29]. GSH levels were evaluated using 90 µL of homogenized supernatant from the liver mixed with monochlorobimane (100 mM) and 10 µL of the catalyst, glutathione S-transferase solution (1 U/mL). The levels of GSH were quantified using a multiscan microplate fluorimeter (FLUOstar optima, BMG Labtech, Offeuburg, Germany) and expressed as the mean ± SEM µmol GSH/g tissue protein for at least three replicates.

2.5 RNA extraction and mRNA quantification by real-time qPCR

The thoracic aorta was homogenized in TissueLyser (Qiagen, Barcelona, Spain), and RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Barcelona, Spain). Total extracted RNA was quantified in a Nanodrop 100 Spectrophotometer (Thermo Scientific, Madrid, Spain). mRNA reverse transcription was carried out by using the High Capacity cDNA Reverse Transcription Kit (Applied

Biosystems, Madrid, Spain). Quantitative PCR amplification and detection were performed in the CFX96 Touch Real Time PCR System (Bio-Rad, Barcelona, Spain) using 96-well plates and SYBR PCR Premix Reagent Ex Taq™ (Takara, Barcelona, Spain) following the commercial protocol.

Relative mRNA levels of eNOS, Arg-1, Klf-2, Sirt-1, NOX4 and ET-1 were analyzed by real-time PCR using glyceraldehyde 3-phosphate dehydrogenase (GADPH) as the housekeeping gene. The primers used for the different genes are shown in Table 1 and were obtained from Biomers (Söflinger, Germany). Primer specificity was verified by melting curve analysis, and the amplicon size was verified by 3% agarose gel electrophoresis. The efficiency of qPCR was calculated by evaluating a 2-fold dilution series of aortic cDNA and calculated by $E = 10^{(1/slope)}$. The results were expressed as the logarithm of the cDNA concentration *vs* the obtained Ct. Relative expression was calculated by dividing the E^{Ct} of the studied gene by the E^{Ct} of the housekeeping gene used as the control and then divided by the value of control, water group gene expression. Each sample was performed in triplicate.

2.6 Experiments in aortic rings

Male Sprague-Dawley (SD) rats, 17–22 weeks old and weighing 240–305 g, were sacrificed by decapitation. To perform the experiment, the thoracic aorta was excised from each of the animals, and excess fat and connective tissue were removed. To obtain the aortic preparations, the tissue was placed in a dissecting dish containing Krebs-Henseleit solution (NaCl, 118 mM: KCl, 4.7 mM; $CaCl_2$, 2.5 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 25 mM; and glucose, 10.0 mM) and cut into 3–4 mm rings. Aortic rings were mounted between two steel hooks in organ baths containing Krebs-Henseleit solution at

37 °C and continuously bubbled with a 95 % O_2 and 5 % CO_2 mixture, which gave a pH of 7.4. An optimal tension of 2 g was applied to all the aortic rings, and the preparations were adjusted every 15 min during the 60–90 min equilibration period before the evaluation of the tested compounds. The isometric tension was recorded by using an isometric force displacement transducer connected to an acquisition system (Protos 5, Panlab). Then, after the equilibration, 80 mM KCl was added to verify their functionality, and when the contraction had reached the steady state (approximately 15 min after the administration), the preparations were washed to recover basal tension.

The presence of endothelium was confirmed by the relaxation in response to the addition of 10 µM acetylcholine of segments previously contracted by treatment with 10^{-5} M methoxamine. A relaxation equal to or greater than 80% was considered evidence of the functional integrity of the endothelium, and the absence of relaxation in response to acetylcholine was considered the absence of endothelium.

The rings were exposed to 10^{-5} M methoxamine to obtain steady state contraction, and AVFQHNCQE curves (10^{-8}–10^{-4} M) were assayed in the methoxamine-pre-constricted rings. Water, as a negative control, was added to the bath at the same volumes used to assay the AVFQHNCQE dose-response curve. The relaxation responses were expressed as a percentage of the pre-contraction induced by methoxamine, which was considered 100 percent. Concentration–dependent response curves were fitted to a logistic equation, and statistical analysis was performed to compare the curves.

All animal protocols followed in this study were approved by the Bioethical Committee of the Universitat Rovira i Virgili (European Commission Directive 86/609) and the Spanish Royal Decree 223/1988.

2.7 Statistical analysis

The results were expressed as the mean ± SEM of six animals per group or at least six experiments including the aortic rings extracted from 6 different animals in the vascular reactivity study. The data from the L-NAME and indomethacin experiments were analyzed by one-way ANOVA (Tukey's test) using IBM SPSS Statistics 20.0 software (SPSS, Inc., Chicago, IL, USA). Plasma ACE activity, GSH content and gene expression results were analyzed by the unpaired Student's t test for independent samples using IBM SPSS Statistics 20.0 software (SPSS, Inc). Differences between concentration–response curves in aortic ring experiments were analyzed by two-way analysis of variance (two-way ANOVA). Outliers were identified and eliminated by using Grubbs' test. Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1 Effects of AVFQHNCQE on blood pressure in SHR treated with L-NAME or indomethacin

The initial values of SBP in the SHR were 205 ± 1.3 mmHg. Figure 2 and Figure 3 show the changes in SBP in SHR groups that were administered water or 10 mg/kg bw of the peptide AVFQHNCQE and that were treated intraperitoneally with saline solution, L-NAME or indomethacin. As expected, the SHR that received water (water + saline group) did not experience changes in their SBP values at 6 h post-administration; however, the oral administration of 10 mg/kg bw of AVFQHNCQE (AVFQHNCQE + saline group) produced a significant decrease in SBP (-31.0 ± 2.5 mmHg; $p \leq 0.05$) (Figure 2 and Figure 3). Nevertheless, when animals receiving the peptide were intraperitoneally treated with L-NAME (AVFQHNCQE + 30 mg/kg of L-

NAME group), the antihypertensive effect of this peptide disappeared and even resulted in a significant increase in SBP when compared to the SBP in the water + saline group (+ 10.1 ± 2.5 mmHg; $p \leq 0.05$). In contrast, the antihypertensive effect of the peptide was also observed in the animals that were also intraperitoneally injected with 5 mg/kg indomethacin (AVFQHNCQE + 5 mg/kg bw of indomethacin group). Interestingly, the SBP reduction in the animals administered peptide and treated with indomethacin, and the SBP decreases in the animals administered peptide and treated with saline were the same: -31.0 ± 1.2 mmHg and -31.1 ± 1.8 mmHg, respectively.

3.2 Effects of AVFQHNCQE on plasmatic ACE activity, liver GSH concentration and endothelial-related gene expression

No differences in plasma ACE activity were found between the group administered water and the group administered peptide (Figure 4). However, after 6 h, AVFQHNCQE administration produced a marked increase in GSH levels when compared to the group that received water (Figure 5). Hepatic GSH production was 70.27 % higher in the AVFQHNCQE-treated group than in the water-treated group.

Peptide administration did not produce any significant changes in the expression of the eNOS, Arg-1, KLF-2 and Sirt-1 genes (Figure 6A) but produced a significant decrease in ET-1 and NOX4 expression (Figure 6B), showing a reduction of 46.12 % and 93.59 % for ET-1 and NOX4, respectively, when compared to the group administered water.

3.3 Effects of AVFQHNCQE in aortic rings

To evaluate the effects of AVFQHNCQE on vascular reactivity, the peptide was studied in methoxamine pre-constricted aortic ring preparations. Figure 7 shows that AVFQHNCQE did not induce relaxation in aortic ring preparations from SD rats.

4. Discussion

Dietary proteins have been demonstrated to be a potential source of bioactive peptides and to exhibit different bioactivities, including antihypertensive activity [30,31]. It has been reported that some chicken proteins can yield peptides with ACEI and antihypertensive properties [32–34]. In this regard, a previous study carried out by our group in SHR [26] demonstrated that treatment with the chicken foot-derived peptide AVFQHNCQE at a dose of 10 mg/kg bw produced a significant decrease in SBP 6 h post-administration. This peptide was identified from the chicken foot hydrolysate Hpp11 obtained after sample pretreatment (100 °C, 1.5 h) and hydrolysis (2 h, 50 °C, pH=7) with the proteolytic enzyme Protamex [26].

Antihypertensive peptides mainly target ACE inhibition; however, the pathophysiology of HTN is complex, and there are other potential targets where these bioactive peptides may exert their specific antihypertensive actions [35]. Because the mechanisms involved in the antihypertensive effect exerted by the chicken foot-derived peptide AVFQHNCQE were unknown, this study aimed to elucidate the mechanisms underlying the *in vivo* antihypertensive effect of the peptide. Specifically, this study evaluates the potential participation of endothelial-relaxing factors in the antihypertensive effect of AVFQHNCQE. Thus, to evaluate the implication of NO in the antihypertensive effect of the peptide, L-NAME, an inhibitor of eNOS

[36], was administered to SHR. NO induces the production of cAMP and consequently increases dilatation and decreases BP [37]. The results presented in this study provide clear proof of the participation of NO in the BP-lowering effect of AVFQHNCQE, as evidenced by the disappearance of its antihypertensive effect in L-NAME-treated rats. Similar results were observed by Kouguchi et al. for a chicken collagen hydrolysate, demonstrating that collagen-derived peptides can exhibit vasoprotective functions via NO production and effectively protect against atherogenesis [38]. Additionally, the NO-mediated antihypertensive effects of other antihypertensive peptides, including those derived from casein or whey proteins, which also exhibited antioxidant effects, were previously described [31]. Interestingly, in this study, it was demonstrated that AVFQHNCQE also exhibited antioxidant effects by increasing the production of hepatic GSH, the main endogenous antioxidant that reduces oxidative stress and free radical damage [39].

In HTN, oxidative stress limits NO bioavailability [2], and the presence and possible involvement of oxidative stress in HTN in SHR has been reported [40,41]. In fact, free radicals in the endothelium can directly scavenge NO and avoid NO-dependent vasodilatation. Thus, considering that the antihypertensive effect of the peptide was NO-mediated, the antioxidant effect produced by this peptide could contribute to the reduction of oxidative stress, consequently increasing NO bioavailability. Similarly, Dávalos et al. also reported antioxidant activity for ACEI egg white protein-derived peptides, suggesting that both mechanisms, the inhibition of ACE and the antioxidant effect, could contribute to the control of HTN [24].Considering that NO was clearly involved in the antihypertensive effect of AVFQHNCQE, whether or not the genes involved in NO production pathway were

overexpressed in animals that received the peptide was evaluated. However, no significant changes in mRNA expression of eNOS, Arg-1, Sirt-1 and KLF-2 were observed. In addition, ET-1 and NOX4 mRNA expression was evaluated after peptide administration. ET-1 is considered the main vasoconstrictor in the endothelium [42], and NOX4 is the main producer of free radicals in the vasculature [21]. In particular, an increase of NOX4 under stressful conditions was previously found to be detrimental [43]. In this study, the administration of AVFQHNCQE reduced the expression of ET-1. Previous studies have demonstrated that the modulation of endothelial ET-1 release was key in the BP-lowering effect of several other antihypertensive peptides, including milk-protein derived peptides [44]. In addition, Fernández-Musoles et al. reported the capacity of lactoferricin B-derived peptides, which demonstrated ACEI properties, to inhibit the endothelin-converting enzyme responsible for ET-1 production [45]. Moreover, AVFQHNCQE was able to reduce the expression of NOX4. A reduction in this enzyme is related to a reduction in ROS production. In fact, it has been demonstrated that a reduction of this enzyme mediates the antihypertensive effect of the commonly used antihypertensive drug atorvastatin [46]. Thus, considering these findings, improved endothelium function contributes to the antihypertensive effect of AVFQHNCQE.

Considering these findings, which demonstrate that AVFQHNCQE decreases BP *via* NO and reduces the expression of ET-1 and NOX4, we expected that this peptide would also be able to induce relaxation in aortic ring preparations. However, the peptide did not relax the aortic ring preparations. Thus, our *in vivo* and *ex vivo* results are not in accordance. Nevertheless, we should not forget that the aorta is a conduit artery, and resistance arteries determine the arterial BP more

than the large vessels [47]. In addition, it should be highlighted that SD rats are normotensive animals and AVFQHNCQE could present different effects in the arteries of hypertensive animals. Therefore, we believe that the vasodilation produced by NO is involved in the antihypertensive effect of the peptide, despite that in this experiment we could not demonstrate the relaxing properties of the peptide.

The endothelium also secretes other vasodilator agents than NO such as PGI_2. Although NO is considered the more important vasodilator in endothelium, PGI_2 was the first endothelium-derived relaxing substance described. This prostaglandin is mainly produced by the cyclooxygenase enzyme (COX), which is also released in the endothelium and is considered a central cardioprotective hormone [48,49]. To evaluate the implication of the vasodilator PGI_2 in the BP-lowering effect of the peptide, the antihypertensive effect was evaluated in rats treated with indomethacin, which is a COX inhibitor. Although some peptides produce PGI_2-mediated antihypertensive effects, such as novokinin, a potent antihypertensive peptide designed based on the structure of ovokinin [50], in this study, indomethacin treatment did not modify the antihypertensive effect of AVFQHNCQE. Therefore, considering this result, the participation of PGI_2 in the antihypertensive effect of AVFQHNCQE could be discarded.

Finally, considering that AVFQHNCQE has shown *in vitro* ACEI activity [26], the *in vivo* effect of this peptide on ACE activity was also studied. However, the *in vitro* ACE inhibitory activity of the peptide did not correspond to the same bioactivity *in vivo* because no changes were observed in the plasma ACE activity of peptide-treated SHR 6 h post-administration when compared to the activity in the water-treated SHR. Although oral administration of ACE inhibitors has been widely used to decrease BP in animal models and humans, it remains unclear

which organs these drugs target to achieve their antihypertensive effects [51]. Moreover, in contrast to the multitude of studies that have addressed the effects of peptide application on BP, a relatively small number of studies have quantified ACEI effects of peptides *in vivo* [52]. Nevertheless, the physiological and pharmacological consequences of the lack of effectivity on ACE *in vivo* do not seem to reduce the therapeutic effect of these drugs [53]. In fact, increased levels of plasma ACE activity have been found in SHR acutely treated with captopril, a known ACE inhibitory drug [54].

5. Conclusions

In this study with SHR, as represented in Figure 8, we demonstrated the participation of NO in the antihypertensive effect exerted by chicken foot-derived peptide AVFQHNCQE. This peptide could enhance NO availability by reducing oxidative stress. In addition, the reduction in ET-1 and NOX4 mRNA expression after peptide administration also contributed to improved endothelium functionality and therefore reduce BP.

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Conflict of interest

No conflicts of interest exist in this manuscript.

References

[1] F.H. Messerli, B. Williams, E. Ritz, Essential hypertension, Lancet. 370 (2007) 591–603.

[2] S. Oparil, M.C. Acelajado, G.L. Bakris, D.R. Berlowitz, R. Cífková, A.F. Dominiczak, G. Grassi, J. Jordan, N.R. Poulter, A. Rodgers, P.K. Whelton, Hypertension, Nat. Rev. Dis. Prim. 4 (2018).

[3] Ž. Reiner, How to improve cardiovascular diseases prevention in Europe?, Nutr. Metab. Cardiovasc. Dis. 19 (2009) 451–454.

[4] G. Antonakoudis, L. Poulimenos, K. Kifnidis, C. Zouras, H. Antonakoudis, Blood pressure control and cardiovascular risk reduction, (2007) 114–119.

[5] A. Quirós, M. del M. Contreras, M. Ramos, L. Amigo, I. Recio, Stability to gastrointestinal enzymes and structure–activity relationship of β-casein-peptides with antihypertensive properties, Peptides. 30 (2009) 1848–1853.

[6] N. Yamamoto, Antihypertensive peptides derived from food proteins, Biopolym. - Pept. Sci. Sect. 43 (1997) 129–134.

[7] F. Hong, L. Ming, S. Yi, L. Zhanxia, W. Yongquan, L. Chi, The antihypertensive effect of peptides: A novel alternative to drugs?, Peptides. 29 (2008) 1062–1071.

[8] S. a Atlas, The renin-angiotensin aldosterone system: pathophysiological role and pharmacologic inhibition., J. Manag. Care Pharm. 13 (2007) 9–20.

[9] Y. Guo, D. Pan, M. Tanokura, Optimisation of hydrolysis conditions for the production of the angiotensin-I converting enzyme (ACE) inhibitory peptides from whey protein using response surface methodology, Food Chem. 114 (2009) 328–333. http://dx.doi.org/10.1016/j.foodchem.2008.09.041.

[10] K. Majumder, S. Chakrabarti, J.S. Morton, S. Panahi, S. Kaufman, S.T. Davidge, J. Wu, Egg-derived ACE-inhibitory peptides IQW and LKP reduce blood pressure in spontaneously hypertensive rats, J. Funct. Foods. 13 (2015) 50–60. http://dx.doi.org/10.1016/j.jff.2014.12.028.

[11] M. Miguel, M. Manso, A. Aleixandre, M.J. Alonso, M. Salaices, R. Lopez-Fandino, Vascular effects, angiotensin I-converting enzyme (ACE)-inhibitory activity, and anti hypertensive properties of peptides derived from egg white, J. Agric. Food Chem. 55 (2007) 10615–10621.

[12] A. Nonaka, T. Nakamura, T. Hirota, A. Matsushita, M. Asakura, K. Ohki, M. Kitakaze, The milk-derived peptides Val-Pro-Pro and Ile-Pro-Pro attenuate arterial dysfunction in L-NAME-treated rats, Hypertens. Res. 37 (2014) 703–707. doi:10.1038/hr.2014.72.

[13] K. Tatemoto, K. Takayama, M.X. Zou, I. Kumaki, W. Zhang, K. Kumano, M. Fujimiya, The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism, Regul. Pept. 99 (2001) 87–92. doi:10.1016/S0167-0115(01)00236-1.

[14] C.C. Udenigwe, A. Mohan, Mechanisms of food protein-derived antihypertensive peptides other than ACE inhibition, J. Funct. Foods. 8 (2014) 45–52.

[15] H.A.R. Hadi, C.S. Carr, J. Al Suwaidi, Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome., Vasc. Health Risk Manag. 1 (2005) 183–98. http://www.ncbi.nlm.nih.gov/pubmed/17319104 (accessed February 14, 2018).

[16] N. Matoba, H. Usui, H. Fujita, M. Yoshikawa, A novel anti-hypertensive peptide derived from ovalbumin induces nitric oxide-mediated vasorelaxation in an isolated SHR mesenteric artery, FEBS Lett. 452 (1999) 181–184. doi:10.1016/S0014-5793(99)00587-6.

[17] W. Durante, F.K. Johnson, R.A. Johnson, Arginase: A critical regulator of nitric oxide synthesis and vascular function, Clin. Exp. Pharmacol. Physiol. 34 (2007) 906–911. doi:10.1111/j.1440-1681.2007.04638.x.

[18] Z. Pons, M. Margalef, F.I. Bravo, A. Arola-Arnal, B. Muguerza, Grape seed flavanols decrease blood pressure via Sirt-1 and confer a vasoprotective pattern in rats, J. Funct. Foods. 24 (2016) 164–172.

[19] G.B. Atkins, M.K. Jain, Role of Krüppel-Like Transcription Factors in Endothelial Biology, Circ. Res. 100 (2007) 1686–1695.

[20] L. Martínez-Fernández, Z. Pons, M. Margalef, A. Arola-Arnal, B. Muguerza, Regulation of Vascular Endothelial Genes by Dietary Flavonoids: Structure-Expression Relationship Studies and the Role of the Transcription Factor KLF-2, J. Nutr. Biochem. 26 (2014) 277–84.

[21] M.J. Zarzuelo, R. López-Sepúlveda, M. Sánchez, M. Romero, M. Gómez-Guzmán, Z. Ungvary, F. Pérez-Vizcaíno, R. Jiménez, J. Duarte, SIRT1 inhibits NADPH oxidase activation and protects endothelial function in the rat aorta: Implications for vascular aging, Biochem. Pharmacol. 85 (2013) 1288–1296.

[22] G. Zalba, F.J. Beaumont, G.S. José, A. Fortuño, A. María, J.C. Etayo, J. Díez, G.S. Jose, A. Fortun, J. Dı, Vascular NADH / NADPH Oxidase Is Involved in Enhanced Superoxide Production in Spontaneously Hypertensive Rats, (2000) 1055–1062.

[23] H. Cai, D.G. Harrison, Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress., Circ. Res. 87 (2000) 840–4. http://www.ncbi.nlm.nih.gov/pubmed/11073878 (accessed October 24, 2018).

[24] C.C. Udenigwe, M.C. Udechukwu, C. Yiridoe, A. Gibson, M. Gong, Antioxidant mechanism of potato protein hydrolysates against in vitro oxidation of reduced glutathione, J. Funct. Foods. 20 (2016) 195–203. doi:10.1016/j.jff.2015.11.004.

[25] R. He, A. Alashi, S.A. Malomo, A.T. Girgih, D. Chao, X. Ju, R.E. Aluko, Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein hydrolysates, Food Chem. 141 (2013) 153–159. doi:10.1016/j.foodchem.2013.02.087.

[26] B. Bravo, F.I; Arola, L.; Muguerza, Procedure for obtaining a hydrolyzate claw chicken leg with antihypertensive activity, and peptides obtained hydrolyzate containing, 2017.

[27] R.D. Buñag, J. Butterfield, Tail-cuff blood pressure measurement without external preheating in awake rats., Hypertension. 4 (n.d.) 898–903.

[28] M. Miguel, M.J. Alonso, M. Salaices, A. Aleixandre, R. López-Fandiño, Antihypertensive, ACE-inhibitory and vasodilator properties of an egg white hydrolysate: Effect of a simulated intestinal digestion, Food Chem. 104 (2007) 163–168. doi:10.1016/j.foodchem.2006.11.016.

[29] H. Kamencic, A. Lyon, P.G. Paterson, B.H.J. Juurlink, Monochlorobimane

Fluorometric Method to Measure, 37 (2000) 35–37.

[30] D. Martínez-Maqueda, B. Miralles, I. Recio, B. Hernández-Ledesma, Antihypertensive peptides from food proteins: A review, Food Funct. 3 (2012) 350–361. doi:10.1039/c2fo10192k.

[31] B. Hernández-Ledesma, M. Del Mar Contreras, I. Recio, Antihypertensive peptides: Production, bioavailability and incorporation into foods, Adv. Colloid Interface Sci. 165 (2011) 23–35. doi:10.1016/j.cis.2010.11.001.

[32] S. Yuliatmo R, Fitriyanto N. A, Bachruddin Z, Jamhari, E. Y., Increasing of angiotensin converting enzyme inhibitory derived from chicken leg, Int. Food Res. J. 24 (2017) 1799–1804. http://www.ifrj.upm.edu.my/24 (04) 2017/(58).pdf.

[33] P. Sangsawad, S. Roytrakul, J. Yongsawatdigul, Angiotensin converting enzyme (ACE) inhibitory peptides derived from the simulated in vitro gastrointestinal digestion of cooked chicken breast, J. Funct. Foods. 29 (2017) 77–83.

[34] M. Terashima, T. Baba, N. Ikemoto, M. Katayama, T. Morimoto, S. Matsumura, Novel angiotensin-converting enzyme (ACE) inhibitory peptides derived from boneless chicken leg meat, J. Agric. Food Chem. 58 (2010) 7432–7436.

[35] K. Majumder, J. Wu, Molecular Targets of Antihypertensive Peptides : Understanding the Mechanisms of Action Based on the Pathophysiology of Hypertension, (2015) 256–283.

[36] S. Moncada, E.A. Higgs, Endogenous nitric oxide: physiology, pathology and clinical relevance, Eur. J. Clincal Investig. 21 (1991) 361–374.

[37] J. Loscalzo, G. Welch, Nitric oxide and its role in the cardiovascular system., Prog. Cardiovasc. Dis. 38 (1995) 87–104.

[38] T. Kouguchi, Y. Zhang, M. Sato, Y. Takahata, F. Morimatsu, Vasoprotective Effect of Foods as Treatments : Chicken Collagen Hydrolysate, (2004).

[39] R.J. Elias, S.S. Kellerby, E.A. Decker, Antioxidant activity of proteins and peptides, Crit. Rev. Food Sci. Nutr. 48 (2008) 430–441. doi:10.1080/10408390701425615.

[40] H. Suzuki, A. Swei, B.W. Zweifach, G.W. Schmid-Schönbein, In vivo evidence for microvascular oxidative stress in spontaneously hypertensive rats. Hydroethidine microfluorography., Hypertens. (Dallas, Tex. 1979). 25 (1995) 1083–9. doi:10.1161/01.HYP.25.5.1083.

[41] H. Negishi, M. Njelekela, K. Ikeda, M. Sagara, T. Noguchi, S. Kuga, T. Kanda, L. Liu, Y. Nara, M. Tagami, Y. Yamori, Assessment of in vivo oxidative stress in hypertensive rats and hypertensive subjects in Tanzania, Africa, Hypertens Res. 23 (2000) 285–289. doi:10.1291/hypres.23.285.

[42] T. Masaki, T. Sawamura, Endothelin and endothelial dysfunction, Proc. Japan Acad. Ser. B. 82 (2006) 17–24. doi:10.2183/pjab.82.17.

[43] A. Konior, A. Schramm, M. Czesnikiewicz-Guzik, T.J. Guzik, NADPH Oxidases in Vascular Pathology, Antioxid. Redox Signal. 20 (2014) 2794–2814. doi:10.1089/ars.2013.5607.

[44] W. Maes, J. Van Camp, V. Vermeirssen, M. Hemeryck, J.M. Ketelslegers, J. Schrezenmeir, P. Van Oostveldt, A. Huyghebaert, Influence of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg (ALPMHIR) on the release of endothelin-1 by endothelial cells, Regul. Pept. 118 (2004) 105–109.

[45] R. Fernández-Musoles, J.J. López-Díez, G. Torregrosa, S. Vallés, E. Alborch, P. Manzanares, J.B. Salom, Lactoferricin B-derived peptides with inhibitory effects on ECE-dependent vasoconstriction, Peptides. 31 (2010) 1926–1933.

[46] W. Cui, K. Matsuno, K. Iwata, M. Ibi, M. Katsuyama, T. Kakehi, M. Sasaki, K. Ikami, K. Zhu, C. Yabe-Nishimura, NADPH Oxidase Isoforms and Anti-hypertensive Effects of Atorvastatin Demonstrated in Two Animal Models, J. Pharmacol. Sci. J Pharmacol Sci. 111 (2009) 260–2683. doi:10.1073/pnas.93.7.2770.

[47] Z. Pons, L. Arola, Involvement of nitric oxide and prostacyclin in the antihypertensive effect of low-molecular-weight procyanidin rich grape seed extract in male spontaneously hypertensive rats, 6 (2013).

[48] J.A. Mitchell, F. Ali, L. Bailey, L. Moreno, L.S. Harrington, Role of nitric oxide and prostacyclin as vasoactive hormones released by the endothelium, Exp. Physiol. 93 (2008) 141–147. doi:10.1113/expphysiol.2007.038588.

[49] S. Moncada, R. Gryglewski, S. Bunting, J.R. Vane, An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation, Nature. 263 (1976) 663–665. doi:10.1038/263663a0.

[50] Y. Yamada, D. Yamauchi, H. Usui, H. Zhao, M. Yokoo, K. Ohinata, M. Iwai, M. Horiuchi, M. Yoshikawa, Hypotensive activity of novokinin, a potent analogue of ovokinin(2–7), is mediated by angiotensin AT2 receptor and prostaglandin IP receptor, Peptides. 29 (2008) 412–418. doi:10.1016/J.PEPTIDES.2007.11.017.

[51] S. Takai, D. Jin, M. Sakaguchi, M. Miyazaki, Significant target organs for hypertension and cardiac hypertrophy by angiotensin-converting enzyme inhibitors., Hypertens. Res. 27 (2004) 213–9. doi:10.1291/hypres.27.213.

[52] M. Martin, A. Deussen, Effects of natural peptides from food proteins on angiotensin converting enzyme activity and hypertension, Crit. Rev. Food Sci. Nutr. 8398 (2017) 1–20. doi:10.1080/10408398.2017.1402750.

[53] O. Costerousse, J. Allegrini, J.P. Clozel, J. Ménard, F. Alhenc-Gelas, Angiotensin I-converting enzyme inhibition but not angiotensin II suppression alters angiotensin I-converting enzyme gene expression in vessels and epithelia, J. Pharmacol. Exp. Ther. 284 (1998) 1180–1187.

[54] M. Miguel, M.A. Manso, P.J. Martín-, A. Aleixandre, R. López-fandiæo, Angiotensin-converting enzyme activity in plasma and tissues of spontaneously hypertensive rats after the short- and long-term intake of hydrolysed egg white, (2007) 555–563.

TABLES

Table 1. List of primers characteristics.

Rat primers	Sequence (5′.....3′)	Size of amplicon	Efficiency	GenBank accession No.
eNOS Fw	GGATTCTGGCAAGACCGATTAC	159	2.06 (100.67%)	NM_021838.2
eNOS Rv	GGTGAGGACTTGTCCAAACACT			
Arg1 Fw	GGCAGTGGCGTTGACCTTGT	158	2.14 (105.64%)	NM_017134.3
Arg1 Rv	AGCAGCGTTGGCCTGGTTCT			
Sirt1 Fw	TTGGCACCGATCCTCGAA	217	2.02 (97.49%)	NM_001007684.1
Sirt1 Rv	ACAGAAACCCCAGCTCCA			
KLF2 Fw	GGACGCACACAGGTGAGAA	186	2.18 (107.85%)	NM_001007684.1
KLF2 Rv	ACATGTGTCGCTTCATGTGC			
ET-1 Fw	TGATTCTCTTGCCTCTTCTTG	110	3.08 (136.04%)	NM_012548.2
ET-1 Rv	TATGGAATCTCCTGGCTCTC			
NOX4 Fw	GTGTCTGCATGGTGGTGGTA	150	2.24 (110.62%)	NM_053524.1
NOX4 Rv	TCAACAAGCCACCCGAAACA			
GADPH Fw	CCATGTTCGTCATGGGTGTG	91	1.97 (98.93 %)	NM_002046.4
GADPH Rv	GGTGCTAAGCAGTTGGTGGTG			

FIGURES

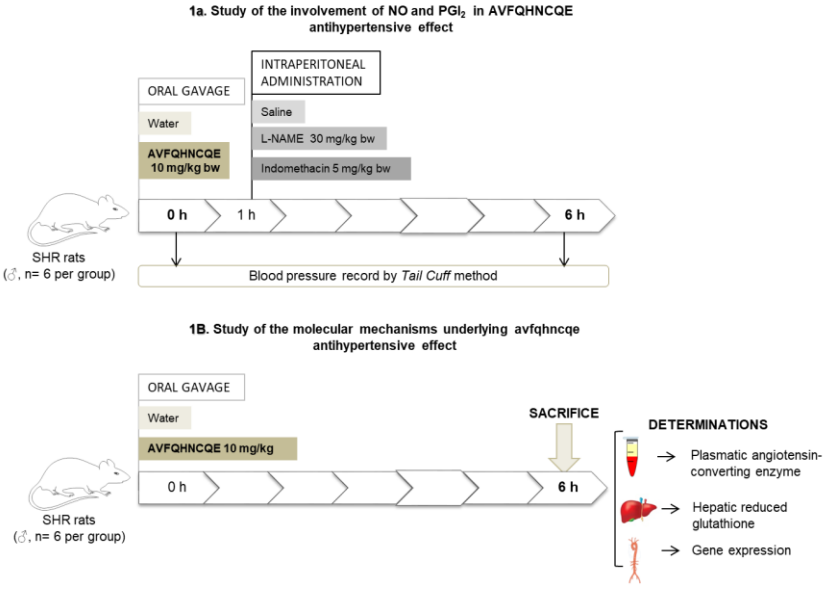

Figure 1. Graphical representation of the experimental design for N_{ω}-nitro-L-arginine methyl ester hydrochloride and indomethacin study in spontaneously hypertensive rats (1A) and graphical representation of the experimental design used to study the effects of peptide administration on the renin-angiotensin-aldosterone system (RAAS), endothelial function and oxidative stress in SHR (1B).

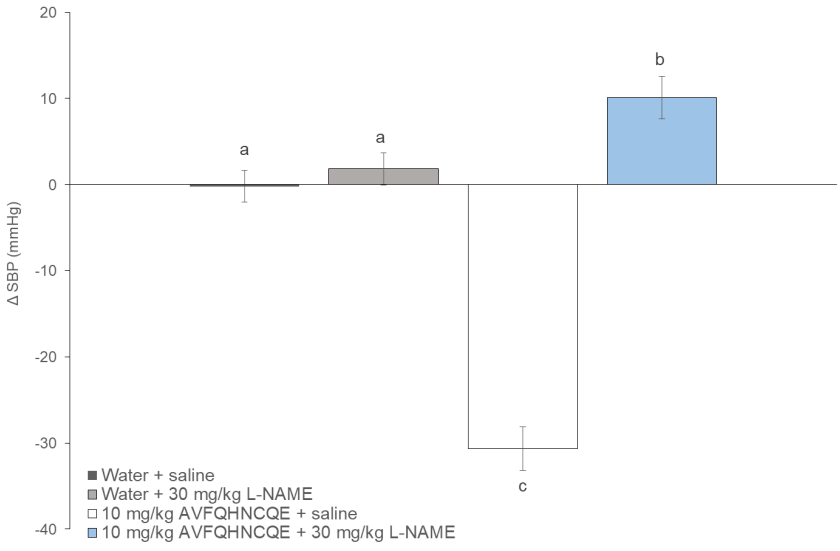

Figure 2. Changes in systolic blood pressure (SBP) caused in spontaneously hypertensive rats after different treatments: oral administration of water + intraperitoneal injection of saline (a), oral administration of water + intraperitoneal injection of 30 mg/kg bw N_{ω}-nitro-L-arginine methyl ester hydrochloride (b), oral administration of 10 mg/kg bw AVFQHNCQE + intraperitoneal injection of saline (c) or oral administration of 10 mg/kg bw AVFQHNCQE + intraperitoneal injection 30 mg/kg bw N_{ω}-nitro-L-arginine methyl ester hydrochloride (b). Data are expressed as the mean ± standard error (SEM) of six animals. Different letters represent statistically significant differences ($p \leq 0.05$). The value of p was estimated by one-way ANOVA.

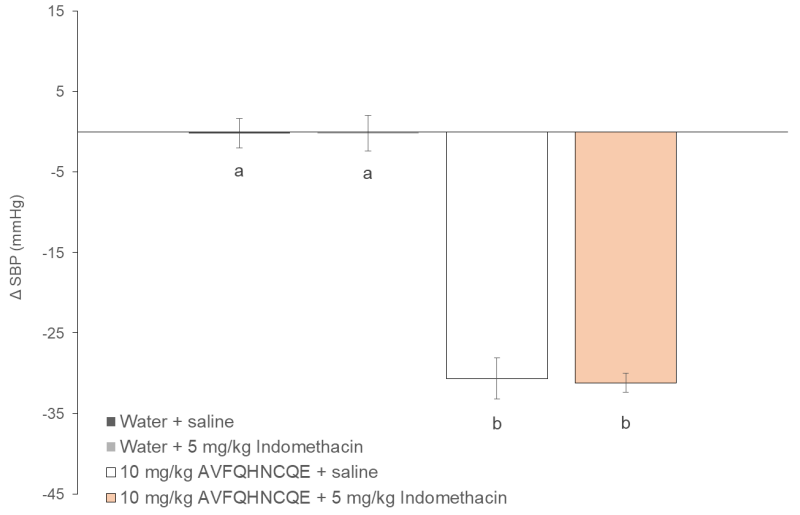

Figure 3. Changes in systolic blood pressure (SBP) caused in spontaneously hypertensive rats after different treatments: oral administration of water + intraperitoneal injection of saline (a), oral administration of water + intraperitoneal injection of 5 mg/kg bw Indomethacin (a), oral administration of 10 mg/kg bw AVFQHNCQE + intraperitoneal injection of saline (b) or oral administration of 10 mg/kg bw AVFQHNCQE + intraperitoneal injection of 5 mg/kg bw Indomethacin bw (b). Data are expressed as the mean ± standard error (SEM) for six animals. Different letters represent statistically significant differences ($p \leq 0.05$). The value of p was estimated by one-way ANOVA.

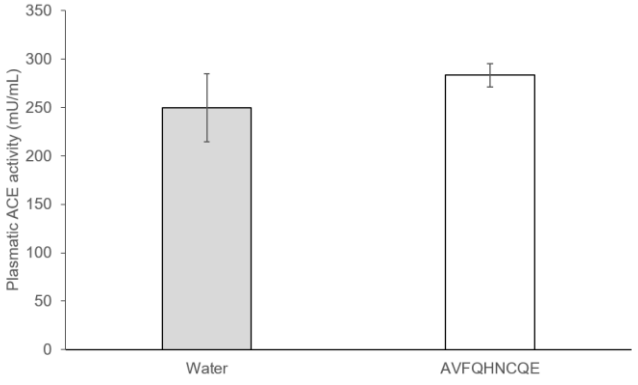

Figure 4. The effect of the peptide AVFQHNCQE on plasma ACE activity 6 h post-administration in spontaneously hypertensive rats. Values are means ± SEM (n=6 animals per group). The results were analyzed with Student's t test, and the differences between the means were considered significant when $p \leq 0.05$. The asterisk indicates statistically significant differences.

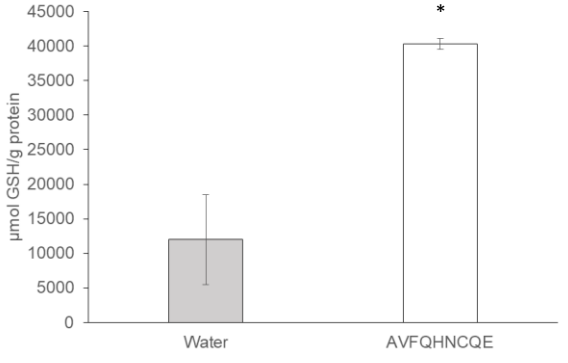

Figure 5. Liver reduced glutathione (GSH) production 6 h post-administration of the peptide AVFQHNCQE in spontaneously hypertensive rats. Data are expressed as the means ± SEM (n=6 animals per group). The results were analyzed with Student's t test, and the differences between the means were considered significant when $p \leq 0.05$. The asterisks indicate statistically significant differences.

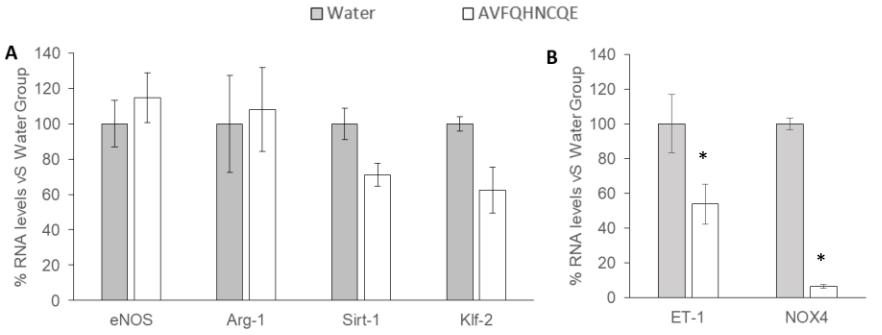

Figure 6. The effect of the peptide AVFQHNCQE on the expression of genes involved in the nitric oxide pathway (A) and on genes related to vasoconstriction and radical oxygen species production (B) in the endothelium at 6 h post-administration in spontaneously hypertensive rats. Values are means (n=6 animals per group) with their standard errors. The results were analyzed with Student's t test, and the differences between the means were considered significant when $p \leq 0.05$. The asterisks indicate statistically significant differences.

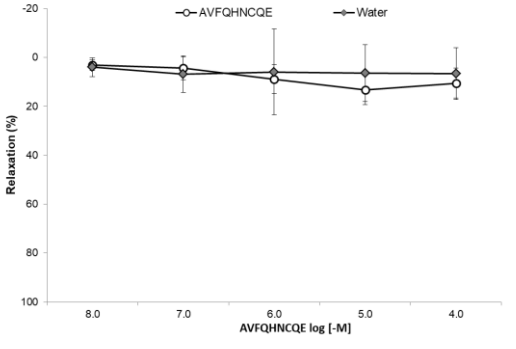

Figure 7. Cumulative concentration–response curves of AVFQHNCQE (10^{-8} - 10^{-4} mg/mL) in methoxamine pre-constricted aortic rings from Sprague-Dawley rats. Water was employed as a control, adding the same volume used to carry out the concentration-response curves of the peptide. Data are mean values ± SEM (n=6 animals per group). No significant differences were observed between the control group and the AVFQHNCQE group.

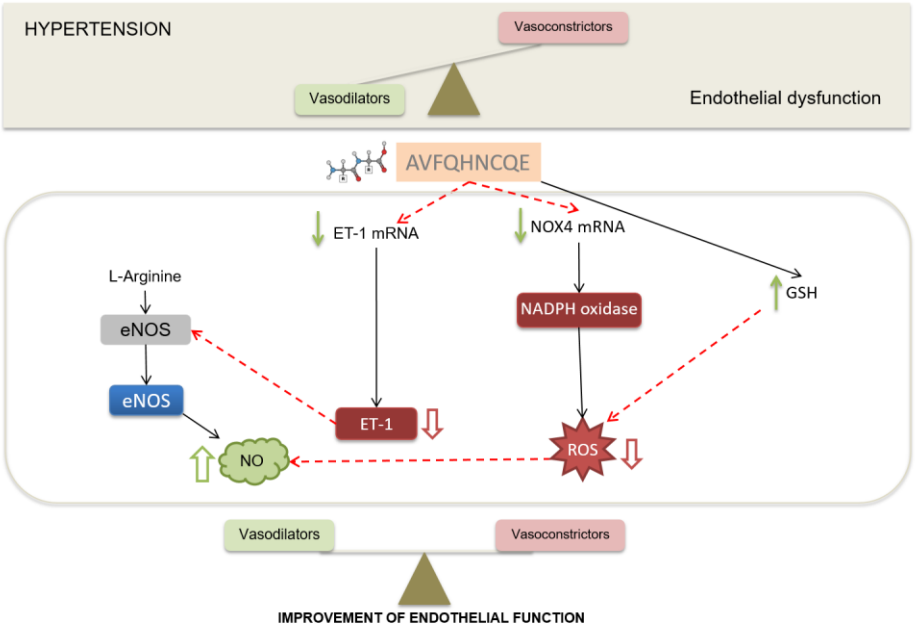

Figure 8. In hypertension endothelial dysfunction is produced. This pathological state is characterized by an imbalance between the vasodilator (mainly NO) and the vasoconstriction (mainly ET-1) endothelial factors. Due to the administration of AVFQHNCQE produced NO-mediated antihypertensive effect it was expected an increase in NO bioavailability. NOX4 mRNA was found decreased in rats administered the peptide leading to a decrease in ROS production and thus, increasing NO availability. The mRNA of ET-1, the most important endothelial vasoconstrictor factor and inhibitor of NO synthesis, was also found decreased. Finally, the peptide increased the production of GSH, the main endogen antioxidant system which reduces ROS and thus, reduces NO scavenging. All this findings lead to enhance nitric oxide, and therefore, reduce the vasoconstriction observed under hypertension conditions.

MANUSCRIPT 6

Objective

To study chicken foot-derived peptide AVFQHNCQE bioavailability and mechanism of action to produce its effect on BP.

Opioid-like activity mediates the antihypertensive effect of the bioactive peptide AVFQHNCQE and clarifies its lack of absorption

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Abstract

The antihypertensive effect of the peptide AVFQHNCQE in spontaneously hypertensive rats (SHR) has been recently demonstrated. This study investigates the bioavailability and the opioid-like activity of this peptide after its oral administration. AVFQHNCQE was administered to male Wistar rats at a dose of 10 mg/kg body weight. Plasma samples were collected before (0 h) and at different time points post-administration. An additional in vitro study using Caco-2 monolayer cells was carried out. Peptide and its potential fragments were quantified by UHPLC-MS/MS and UHPLC-HRMS. Both in vitro and in vivo experiments demonstrated that peptide AVFQHNCQE was not absorbed. An additional in vivo experiment using SHR was conducted to test if peptide antihypertensive effect could be mediated through binding intestinal opioid receptors. Naloxone, an opioid antagonist, was intraperitoneal administered to block opioid receptors and the blood pressure (BP) lowering properties of the peptide were evaluated under these conditions. No changes in BP were recorded in rats administered Naloxone, demonstrating that AVFQHNCQE antihypertensive effect is mediated through its interaction with opioid receptors. AVFQHNCQE opioid-like activity would clarify the antihypertensive properties of AVFQHNCQE in spite of its lack of absorption.

Keywords: bioactive peptide, endothelial dysfunction, blood pressure, bioactivity, nitric oxide.

Abbreviations: **ACE,** angiotensin-converting enzyme; **HTN**, hypertension; **BP**, blood pressure; **SHR**, spontaneously hypertensive

rats; **SBP**, systolic blood pressure; **RAAS**, renin angiotensin aldosterone system; **GI,** gastrointestinal.

Chemical compound studied in this article: Naloxone (PubChem CID: 5284596), heparin (PubChem CID: 772) and captopril (PubChem CID: 44093).

1. Introduction

Bioactive peptides are specific protein fragments released by limited proteolysis of their specific precursor proteins, which present additional biological activities over and above their expected nutritional [1,2]. Different health benefits have been reported for these peptides, including antioxidant, antithrombotic, antimicrobial, opioid and anticancer activities [3]. Nevertheless, one of the most important biological property attributed to bioactive peptides is their antihypertensive effects [4]. These peptides are usually obtained and selected by their capacity to inhibit angiotensin-converting enzyme (ACE), key enzyme in blood pressure (BP) regulation [5]. Hypertension (HTN) is a global public health problem [6] and although its medical treatment is well established, it can present side-effects in some patients [7]. Thus, the use of antihypertensive peptides as an alternative for HTN prevention and treatment is receiving interest despite the fact that these inhibitory peptides present lower ACE inhibitory (ACEI) activity than the antihypertensive drugs [4].

Other mechanisms than the inhibition of ACE could be involved in bioactive peptide BP-lowering effect, including an improvement in endothelial function by the activation of nitric oxide (NO) pathway [8]. In this sense, NO has also been reported to mediate the endothelium-dependent vasorelaxation effects of different antihypertensive peptides [8]. The antihypertensive peptides that mediate its effect through NO could enhance NO production by the activation of endothelin NO synthase by phosphorylation at Ser-1177 residue consistent [9]. Additionally, some bioactive peptides also present antioxidant effects, which in turn would contribute to enhance NO

bioavailability and to improve endothelium functionality by reducing radical oxygen species, that are known to be NO scavengers [10].

Peptide *in vivo* physiological effects depend on their ability to reach the target sites in an active form [11]. In this context, gastrointestinal (GI) digestion plays a key role in the formation and degradation of bioactive peptides [12]. Once ingested, peptides might be subjected to hydrolysis by different GI enzymes such as pepsin, trypsin, chymotrypsin and peptidases from the surface of epithelial cells, which could result in the release of different amino acid sequences [13]. In addition to digestion, absorption may also condition peptide bioactivity. In this sense, peptides are absorbed depending on their size and structure. Di- and tri-peptides are frequently transported through PepT1 peptide transporter [14], while larger peptides can be absorbed by passive diffusion [15]. Moreover, water-soluble large peptides can cross via the tight junctions between cells and highly lipid-soluble peptides appear to be able to diffuse via the transcellular route [14].

However, some bioactive peptides might mediate their physiological effects through its binding to receptors present in the intestinal wall, implying that their absorption is not required [13]. In fact, it has been demonstrated that some opioid peptides are not absorbed and can produce their biological effects by the interaction with opioid receptors (μ, δ and κ), which have been described in several tissues including in GI tract [15,16]. In this regard, some opioid peptides have demonstrated to regulate BP and produce its BP-lowering effect through the binding to opioid receptors present in the GI tract [17]. Nurminen et al. [18] evidenced the involvement of opioid receptors in the antihypertensive effect of the peptide α-lactorphin (YGLF), since its BP-lowering effect was abolished by the administration of the non-

selective opioid receptor antagonist, Naloxone [19]. Furthermore, Sipola et al. [20] demonstrated α-lactorphin produced its opioid-mediated antihypertensive effect through NO release.

AVFQHNCQE is a nonapeptide initially identify in the chicken foot hydrolysate Hpp11, which exhibited antihypertensive properties after its oral administration to spontaneously hypertensive rats (SHR) [21]. However, AVFQHNCQE vulnerability to GI enzymes and posterior absorption has not been studied yet. Thus, the aim of this study was to investigate the bioavailability of the antihypertensive peptide AVFQHNCQE after its oral administration. For this purpose, *in silico* and *in vitro* approaches were used to study the peptide susceptibility to GI digestion. In addition, AVFQHNCQE absorption was studied both *in vitro* by using Caco-2 cell monolayers and *in vivo* evaluating peptide presence in plasma from Wistar rats [14]. Finally, a potential involvement of opioid receptors in the BP-lowering effect of AVFQHNCQE was studied in presence of opioid receptors-antagonist Naloxone.

2. Materials and methods

2.1. Materials

The peptide AVFQHNCQE was synthetized by CASLO Aps. (Kongens Lyngby, Denmark) and its purity was 98.94 %. Captopril was provided by Santa Cruz Biotechnology (Dallas, TX, United States). Pepsin, bile salts mixture, trypsin, α-chymotrypsin, porcine pancreatic lipase, colipase, trifluoroacetic acid (TFA), formic acid (FA) LC-MS grade and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Madrid, Spain). Acetonitrile and methanol LC-MS grade were

purchased from Merck (Darmstadt, Germany). The synthetic isotopically labelled peptide IFV*TGQDYNDK (V*= Val-13C5,15N) was obtained from Biomatik (Ontario, Canada) and used as internal standard (IS) for MS experiments. Chicken foot hydrolysate Hpp11 was obtained by our group following the procedure described by Bravo et al. [21,22] . All other chemical solvents used were of analytical grade.

2.2. *In silico and in vitro* simulated digestion

In silico simulated peptide digestion was carried out using the programs ExPASy PeptideCutter, available at http://web.expasy.org/peptide_cutter. This approach can predict the hydrolysis of a protein sequence obtained from a protein database using the known enzymatic cleavage sites. The digestive enzymes pepsin, chymotrypsin, trypsin, lipase and colipase were used to predict the protein fragments generated in GI digestion of AVFQHNCQE.

For the *in* vitro simulated peptide GI digestion, two-stage hydrolysis process were carried out according to Martos et al. [23]. AVFQHNCQE was dissolved in simulated gastric fluid (35 mM NaCl at pH 2 for 15 min) and posteriorly subjected to digestion by porcine pepsin (E.C. 3.4.23.1, 3440 units/mg) at an enzyme/substrate ratio of 1:20 w/w at 37 ºC for 60 min. Gastric digestion with pepsin was stopped by adding 1 M $NaHCO_3$ and pH was adjusted to 7.0 with NaOH 1 M (final protein concentration of 5 mg/mL). Aliquots of this gastric digest was collected (G60) and stored at -20 °C until analysis. Duodenal digestions were performed by using, as the starting material, the obtained gastric digests, with the addition of 1M $CaCl_2$, 0.25M bis-Tris pH 6.5, 0.25 M bis-Tris pH 6.5, and a 0.125 M bile salts mixture containing equimolar quantities of sodium taurocholate and glycodeoxycholic acid. Posteriorly, the duodenal enzymes trypsin (EC

232-650-8, 10100 BAEE units/mg protein), α-chymotrypsin (EC 232-671-2; 55 units/mg protein), porcine pancreatic lipase (EC 232-619-9) and colipase (EC 259-490-1), prepared in 35 mM NaCl adjusted to pH 7, were added to the solution. The duodenal digestion was carried out at pH 7, 37 °C during 60 (D60). Then, enzymes were inactivated by heating at 95 °C for 10 min in a water bath, followed by cooling to room temperature. A triplicate of aliquots at each time point were collected and stored at -20 °C until analysis.

2.3. Peptide bioavailability studies

2.3.1. *In vivo* experiment

Male Wistar rats (17-20 weeks-old, weighting between 230 and 250 g) were obtained from Charles River Laboratories (Barcelona, Spain). Animals were maintained in pairs, at 22 °C with light/dark cycles of 12 h and were fed standard chow diet (AO4, Panlab, Barcelona, Spain) *ad libitum* during all the experiment. Animals were randomly divided and administered tap water (control group, n = 6) or 10 mg/kg body weight (bw) peptide (AVFQHNCQE group, n = 6) by gastric intubation after a starvation of 12 h. The total volume of water or AVFQHNCQE orally administered to the rats was 1 mL. Blood samples were collected via saphenous vein extraction by use of heparin vials (Starsted, Barcelona, Spain) before the water or peptide administration and 30 min and 60 min post-administration. Plasma samples were obtained by blood centrifugation (2,000 x g, 15 min, 4 °C) and were pooled (n= 6 per treatment group) to perform UHPLC-MS/MS analysis. Plasma was stored at −80 °C until the analysis.

2.3.2. *In vitro* experiment

Caco-2 cells were obtained from Sigma-Aldrich and were grown Dulbecco's modified minimum essential medium (DMEM), supplemented with 20 % fetal bovine serum (FBS), 2 mmol/mL L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C in and 5 % CO_2. Cells were seeded onto Transwell inserts permeable membrane support (0.4 µm pore, 24 mm diameter, 4.7 cm^2 grown surface from, Merck Co., Bedford, MA) placed in six-well plates. The seeding density was 12,000 cell/cm^2. Medium was replaced every 2-3 days and cells were growing for 21 days. At 21st day of the procedure, the integrity of the Caco-2 monolayer was assessed by the measurement of transepithelial electrical resistance (TEER) using a Millicell ERS-2 voltammeter (EMD Millipore, Darmstadt, Germany) selecting the ones with TEER values higher than 200 Ω/cm^2.

For the transport study, Caco-2 cells, maintained in DMEM, were gently rinsed with Hank's balanced salt solution (HBSS) and equilibrated for 20 min at 37 °C prior to the transport study. Then, it was evaluated the transepithelial transport of the peptide AVFQHNCQE (1mM) dissolved in HBSS and the digests obtained in the *in vitro* simulated digestion (G60 and D60). AVFQHNCQE or the peptide digests G60 and D60 were added to the apical (AP) chamber and were incubated for 1 h at 37 °C. After this time, triplicates of the AP chamber content and the basolateral (BL) chamber content were taken and stored at −80 °C until UHPLC-HRMS analyses.

2.4. Peptide analysis by UHPLC-MS/MS and UHPLC-HRMS

2.4.1 Optimisation of peptide extraction in plasma

In order to efficiently extract the peptide or peptide fragments from plasma after AVFQHNCQE administration in Wistar rats, three different extraction methods were assayed.

Solid phase extraction (SPE): plasma samples (150 µL) were mixed with 25 µL of IFV*TGQDYNDK (IS) (10 ppm) and 800 µL of H_2O (1% TFA). Then, these solutions were heated at 95 °C for 2 min to denature plasma proteins. After cooling down, samples were centrifuged (15,000 rpm, 15 min, 4 °C) and loaded to Oasis HLB (30 mg, 1 mL) cartridges (Waters, Barcelona, Spain), which were sequentially pre-conditioned with 1 mL acetonitrile: water 0.1% FA (75:25, v/v) and 1 mL water 0.1% FA. Loaded cartridges were washed with 1 mL water 0.1% FA and dried. Retained peptides were eluted with 2 sequential additions of 250 µL acetonitrile: water 0.1% FA (75:25, v/v). Eluted samples were totally dried in a speed-vac concentrator (Thermo Fisher, Waltham, Massachusetts), USA), reconstituted in 100 µL of water (0.1 % FA and 0.1 % BSA) and analyzed by UHPLC-MS/MS. The extraction was performed in triplicate.

Protein precipitation by TFA: 150 µL of plasma were mixed with 25 µL of IS (10 ppm) and 40 µL of water (10% TFA) and heated for 2 min at 95 °C to precipitate plasma proteins. After cooling down, samples were centrifuged (15,000 rpm, 15 min, 4 °C) and supernatants were analyzed by UHPLC-MS/MS. The extraction was performed in triplicate.

Protein precipitation by TFA and SPE: plasma samples (150 µL) were mixed with 25 µL of IS (10 ppm) and 40 µL of water (10% TFA) and

heated at 95 °C for 2 min. After cooling down, samples were centrifuged (15,000 rpm, 15 min, 4 °C) and supernatants were purified using Oasis HLB (30 mg, 1 mL) cartridges (Waters) with the same procedure described before and analyzed by UHPLC-MS/MS. The extraction was performed in triplicate.

2.4.2. Peptide extractions from in vitro digestions and Caco-2 monolayers

Samples obtained from *in vitro* peptide simulated digestion and Caco-2 monolayers were diluted 10 times with water (0.1% FA) and centrifuged (15,000 rpm, 15 min, 4°C). Supernatants were directly analyzed by UHPLC-HRMS to avoid the loss of polar peptide fragments. Extensive clean-up or pre-concentration procedures were not necessary due to the high concentration of peptide and low matrix complexity than plasma samples.

2.4.3. Analysis by UHPLC-MS/MS and UHPLC-HRMS

The obtained purified solutions from plasma and peptide digests G60 and D60 and from AP and BL chambers in Caco-2 experiment were analysed using an UHPLC 1290 Infinity II Series coupled to a QqQ 6490 Series (Agilent Technologies, Santa Clara, CA, USA) for tandem mass experiments or a qTOF 6550 Series (Agilent Technologies) for high-resolution mass spectrometry experiments. Chromatographic separation of peptide or their fragments were performed using a Kinetex 2.6 µm EVO C18 column (150×2.1 mm, 2.7 µm) from (Phenomenex, Torrance, USA) as stationary phase and water containing 0.1% formic acid (solvent A) and acetonitrile (solvent B) as mobile phase. Separation was performed in gradient elution as follows: isocratic; 0–0.5 min, 2 % B; 0.5–1 min, 2- 6% B; 1– 5 min, 6- 15% B; 5– 6 min, 15- 35 % B; 6- 10 min, 35-98 % B; 10- 12 min, 98 % B isocratic; 12-

12.10, 98- 2% B. For all runs, 10 µL of sample (4 °C) was injected, and flow rate was 0.350 mL/min and column temperature was 25 °C.

Peptide ionisation was performed in positive electrospray ionization (ESI) and source parameters were as follows and the same for both mass spectrometer instruments: gas temperature 150 °C; gas flow 13 L/min, sheath gas temperature 250 °C; sheath gas flow 11 L/min; nebulizer pressure 40 psi capillary voltage 3000 V and nozzle voltage 500 V. For plasma samples, QqQ instrument was used to quantify AVFQHNCQE peptide because low concentration was expected. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode and optimized transitions were 538.4 > 928.9 (10 eV) for quantification and 538.4 > 758.5 (10 eV) and 538.4 > 453.4 (10 eV) for confirmation (RT = 4.25 min) of AVFQHNCQE and 653.4 > 940.8 (20 eV) for quantification and 653.4 > 1046.0 (20 ev) and 653.4 > 839.7 (25 eV) for confirmation (CE = 20 eV, RT = 6.15 min) of IFV*TGQDYNDK (IS). Peptide quantification was performed by an internal standard calibration curve using the pure standard of AVFQHNCQE peptide with a limit of detection of 0.05 ppb and limit of quantification of 0.130 ppb in plasma and a linear range up to 166 ppb (r^2>0.9999).

For *in vitro* simulated digestion experiments and Caco-2 monolayer samples, qTOF instrument was used to investigate the presence of unknown peptide fragments and previously *in silico* predicted peptide fragments by means of its accurate mass. Peptide and its fragments were semi-quantified using the chromatographic peak area observed in the extracted ion chromatograms using a 20 ppm mass window.

2.5. Evaluation of the opioid-like activity of AVFQHNCQE in SHR

Male, 17–20-week-old, SHR, weighing 286 ± 4 g were used in this study. All these animals were obtained from Charles River Laboratories Spain. The SHR were housed at a temperature of 23 °C with 12 h light/dark cycles, and had free access to tap water and a standard diet (A04 Panlab). Before the experiments, rats were submitted to a training period of 10 days to guarantee the reliability of the measurements. To evaluate if AVFQHNCQE presented opioid-mediated effect, SHR received via subcutaneous Naloxone (3 mg/kg bw), and afterwards a single oral dose (1 mL) of AVFQHNCQE (10 mg/kg bw). Additionally, water, Captopril (50 mg/kg bw), known antihypertensive drug, and chicken foot hydrolysate Hpp11 (55 mg/kg bw), were administered as controls. Captopril and Hpp11 are able to inhibit ACE *in vivo*. All treatments were administered by oral gavage between 8:00 and 9:00 h a.m. The total volume orally administered to the rats for all treatments was of 1 mL. Systolic blood pressure (SBP) was recorded in the rats by the tail cuff method [24] before and 2, 4 and 6 h post-administration. All measurements were taken by the same person in the same peaceful environment.

The Animal Ethics Committee of University Rovira i Virgili approved all procedures (reference number 8868 by Generalitat de Catalunya). All the above-mentioned experiments were performed as authorised (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food, Madrid, Spain).

2.6. Statistical analysis

Results from transepithelial transport studies are presented as means ± standard error mean (SEM). Means were compared using Student's t-test. The results for the experiments investigating the participation of opioid receptors in the AVFQHNCQE, Captopril and Hpp11 antihypertensive effect were expressed as the mean ± SEM of six animals per group (heterogeneity was tested by Levene's test) and were analysed by one-way ANOVA (Tukey's test) using IBM SPSS Statistics 20.0 software. Outliers were identified and eliminated by using Grubbs' test. Differences between groups were considered significant when $p < 0.05$.

3. Results and discussion

Food proteins contain specific peptide sequences that are inactive as long as they remain bonded to other amino acids within the primary protein structure, which might be released by protein hydrolysis [25]. These peptides can present a wide range of bioactivities including ACEI and antihypertensive activity [25]. In this sense, it has been demonstrated that the hydrolysis of chicken foot proteins is a good strategy for the obtaining of bioactive peptides able to reduce BP [21,22]. In fact, the antihypertensive effect of the peptide AVFQHNCQE (10 mg/kg bw), identified initially in the chicken foot hydrolysate Hpp11, has been previously demonstrated in SHR [21]. Nevertheless, it is unknown if this peptide sequence is susceptible to GI digestion, resulting in new peptide fragments which might be the responsible of the AVFQHNCQE bioactivity. Therefore, the peptide fragmentation under GI digestion of the antihypertensive peptide was investigated using *in silico* and *in vitro* approaches. Simulated *in silico* digestion

considers the primary structure of peptides and the known cleavage specificity of the GI tract enzymes, predicting the peptide potential to release other shorter peptides sequences during digestion. Table 1 shows the amino acid sequences of peptides predicted to be released after AVFQHNCQE gastric and duodenal digestion based on the *in silico* digestion approach. This model predicted a peptide hydrolysed only by pepsin and chymotrypsin. Trypsin was also evaluated showing no effects on peptide hydrolysis. Although *in silico* approach is important for the identification of the potential bioactive amino acid sequences, the results of these studies have to be interpreted with caution, since this approach does not consider the tertiary structure of the proteins. However, based on the *in silico* prediction it would appear that AVFQHNCQE could release different amino acid sequences, which could be involved in its antihypertensive effect.

Simulated *in vitro* gastrointestinal digestion model was used to investigate the releasing of the amino acid sequences previously predicted in the *in silico* approach. The peptide was sequentially digested under physiological conditions and the identification of amino acid sequences in samples obtained from both gastric and intestinal digestion were performed by UHPLC-HRMS (Table 2). It was observed in sample G60 the native peptide and also the presence of different peptide fragments. However, the most intense signal corresponded to the native peptide, AVFQHNCQE, indicating that this peptide presents a high resistance to gastric digestion. In this sense, Ruiz et al. reported for the peptides VRYL and KKYNVPQL, derived from Manchego cheese, their resistance to GI digestion and their ability to reach their target organs as an intact form. Interestingly, KKYNVPQL, such as AVFQHNCQE, contains glutamine residue (Q) in the C-terminal, which is commonly presented in peptides resisting GI digestion hydrolysis

[26]. Regarding to duodenal digests, the most intense signal after 60 min under simulated intestinal digestion (D60) corresponded to free amino acids. Taking into account that most antihypertensive peptides require to be absorbed in the intestine to produce their BP-lowering effect [11], absorption of the AVFQHNCQE or their potential derived fragments should produce before 60 min of intestinal digestion.

Therefore, an additional *in vivo* study was carried out in which the presence of the AVFQHNCQE or their fragments were evaluated by chromatographic analysis in Wistar rats plasma collected before and 30 and 60 min post-administration of AVFQHNCQE (10 mg/kg bw) or water. However, since no specific and optimised peptide extraction methodology exist for AVFQHNCQE using plasma samples, this procedure was optimised and validated to obtain the maximum recovery and reproducibility. Three different extraction methodologies (SPE, TFA and SPE+TFA) were evaluated using samples spiked with 1 ppb of AVFQHNCQE peptide to determine recovery (%), repeatability (% RSD) and matrix effect (%). SPE method was demonstrated to be the most efficient method for the peptide extraction in plasma obtaining a recovery of 92.4 % with good reproducibility (Table 3) and negligent matrix effect (less than 20 %). Once the peptide extraction method was optimised, rat plasma at 0, 30 and 60 min after peptide (10 mg/kg bw) or water oral administration were subjected to SPE extraction and analysed by UHPLC-MS/MS for AVFQHNCQE peptide quantification or, by UHPLC-HRMS for peptide fragments analysis. Interestingly, neither the peptide nor their potential fragments were identified in the plasma samples studied, indicating that none of them was absorbed.

In order to verify the lack of absorption of the peptide or their resultant fragments in the GI tract an additional study using Caco-2 cells was

carried out. The native peptide AVFQHNCQE and the samples collected after the simulated gastric and duodenal digestions (G60 and D60, respectively) were incubated in Caco-2 monolayer for 1 h. The peptide and its resultant fragments were identified in the AP chamber from G60 samples, however only a 0.10-0.25 % were transported to the BL chamber (Table 4). Therefore, the presence of native AVFQHNCQE in AP chamber after 1 h of incubation and the absence of the peptide or their fragments in BL chamber demonstrated that it was not absorbed through the intestinal monolayer. Similarly, Miguel et al. reported for the antihypertensive peptide RADHP the lack of transported through Caco-2 monolayer [12].

The absence of the peptide AVFQHNCQE or their potential derived fragments in the mass spectrometry data from the plasma or BL chamber samples suggest that AVFQHNCQE can exert its antihypertensive effect without requiring to be absorbed, by interacting with receptors present in the intestinal tract, as has been previously reported for other large peptides [15]. In this sense, it has been demonstrated that some antihypertensive peptides that also present opioid activity do not require to be absorbed to induce BP reduction. These opioid peptides induce antihypertensive effect by their interaction with the opioid receptors present in the GI tract [27]. The mechanism of action proposed driven by the stimulation of these opioid receptors is the subsequent NO release causing vasodilation [28–31]. Therefore, considering that AVFQHNCQE was not absorbed, an antihypertensive effect mediated by the interaction with opioid receptors present in the intestinal tract was investigated. Thus, to evaluate the involvement of opioid receptors in the antihypertensive properties of AVFQHNCQE, its BP-lowering effect was studied in presence of Naloxone, which is an opioid receptor antagonist [32].

Figure 1, 2 and 3 show the changes in SBP 6 h after oral administration of the peptide, the chicken foot hydrolysate Hpp11 and Captopril in rats previously subcutaneously treated with Naloxone or saline. The results of this study showed that the antihypertensive effect of AVFQHNCQE was completely abolished in Naloxone-treated rats while in the control rats, subcutaneously treated with saline, it was observed −31 ± 2 mmHg of SBP decrease when compared to water group (Figure 1). Similarly to our results, Nurminen et al. reported for the milk-derived peptide α-lactorphin opioid mediated antihypertensive effect [18]. Therefore, considering the present findings, AVFQHNCQE produced its antihypertensive effect through the interaction with opioid receptors, clarifying the reason why this peptide do not require to be absorbed to produce its physiological effect.

Interestingly, Hpp11 chicken foot hydrolysate, in which was initially identify the peptide AVFQHNCQE, and Captopril, an ACEI antihypertensive drug [33], were able to reduce BP in both groups, in the Naloxone-treated group and saline-treated group indicating that their BP-lowering effects were not mediated through the interaction with opioid receptors (Figure 2 and Figure 3). According to this, Hpp11 was demonstrated to produce its antihypertensive effect by reducing plasma ACE activity [22], confirming that as ACE inhibitor, such as Captopril, its effect is not mediated by opioid receptors and would be absorbed and transported to reach the cardiovascular system and to inhibit ACE [34].

4. Conclusions

In the present study, it was demonstrated that AVFQHNCQE showed a high resistance to gastric digestion but it was totally hydrolysed to free amino acids after 60 min in the duodenum. In addition, this peptide did not cross through intestinal epithelium, indicating that AVFQHNCQE does not require its absorption to produce its BP-lowering effect. Indeed, it was demonstrated that the peptide produces its antihypertensive effect through their interaction with opioid receptors. Future studies are required to demonstrate its antihypertensive effect in humans. Nevertheless, this study represents a valuable contribution to clarify the mechanisms implicated in the antihypertensive effect of AVFQHNCQE.

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References

[1] Yoshikawa M. Bioactive peptides derived from natural proteins with respect to diversity of their receptors and physiological effects. Peptides 2015;72:208–25. doi:10.1016/j.peptides.2015.07.013.

[2] Sharma S, Singh R, Rana S. Bioactive peptides: A review. Int J Bioautomation 2011;15:223–50. doi:10.1093/fqsafe/fyx006.

[3] Sánchez A, Vázquez A. Bioactive peptides: A review. Food Qual Saf 2017;1:29–49.

[4] Hong F, Ming L, Yi S, Zhanxia L, Yongquan W, Chi L. The antihypertensive effect of peptides: A novel alternative to drugs? Peptides 2008;29:1062–71.

[5] Margalef M, Bravo FI, Arola-Arnal A, Muguerza B. Natural angiotensin converting enzyme (ACE) inhibitors with antihypetensive properties. In: Andrade P., Valentao P, Pereira DM, editors. Nat. Prod. Target. Clin. Relev. Enzym. First, Weinheim: Wiley-VCH Verlag GmbH & Co. ; 2017, p. 45–67.

[6] Oparil S, Acelajado MC, Bakris GL, Berlowitz DR, Cífková R, Dominiczak AF, et al. Hypertension. Nat Rev Dis Prim 2018;4.

[7] Sica DA. Angiotensin-converting enzyme inhibitors side effects--physiologic and non-physiologic considerations. J Clin Hypertens (Greenwich) 2004;6:410–6.

[8] Udenigwe CC, Mohan A. Mechanisms of food protein-derived antihypertensive peptides other than ACE inhibition. J Funct Foods 2014;8:45–52.

[9] Barba de la Rosa AP, Barba Montoya A, Martínez-Cuevas P, Hernández-Ledesma B, León-Galván MF, De León-Rodríguez A, et al. Tryptic amaranth glutelin digests induce endothelial nitric oxide production through inhibition of ACE: Antihypertensive role of amaranth peptides. Nitric Oxide - Biol Chem 2010;23:106–11. doi:10.1016/j.niox.2010.04.006.

[10] Chakrabarti S, Wu J. Bioactive peptides on endothelial function. Food Sci Hum Wellness 2016;5:1–7. doi:10.1016/J.FSHW.2015.11.004.

[11] Vermeirssen V, Camp J Van, Verstraete W. Bioavailability of angiotensin I converting enzyme inhibitory peptides. Br J Nutr 2004;92:357.

[12] Miguel M, Dµvalos A, Manso MA, Peæa G De, Lasunción MA, López-fandiæo R. Transepithelial transport across Caco-2 cell monolayers of antihypertensive egg-derived peptides . PepT1-mediated flux of Tyr-Pro-Ile 2008:1507–13.

[13] Hernández-Ledesma B, Del Mar Contreras M, Recio I. Antihypertensive

peptides: Production, bioavailability and incorporation into foods. Adv Colloid Interface Sci 2011;165:23–35. doi:10.1016/j.cis.2010.11.001.

[14] Vermeirssen V, Camp J Van, Verstraete W. Bioavailability of angiotensin I converting enzyme inhibitory peptides. Br J Nutr 2004;92:357.

[15] Miner-Williams WM, Stevens BR, Moughan PJ. Are intact peptides absorbed from the healthy gut in the adult human? Nutr Res Rev 2014;27:308–29. doi:10.1017/S0954422414000225.

[16] Holzer P. Opioid receptors in the gastrointestinal tract. Regul Pept 2009;155:11–7. doi:10.1016/j.regpep.2009.03.012.Opioid.

[17] Pihlanto-leppa A. Bioactive peptides derived from bovine whey proteins : opioid and ace- inhibitory peptides. J. Food Sci. Technol. 2001;11: 347-356.

[18] Nurminen ML, Sipola M, Kaarto H, Pihlanto-Leppälä A, Piilola K, Korpela R, et al. Alpha-lactorphin lowers blood pressure measured by radiotelemetry in normotensive and spontaneously hypertensive rats. Life Sci 2000;66:1535–43.

[19] Levin ER, Mills S, Weber MA. Endogenous opioids and opiate antagonists modulate the blood pressure of the spontaneously hypertensive rat. Peptides 1986;7:977–81. doi:10.1016/0196-9781(86)90124-5.

[20] Sipola M, Finckenberg P, Vapaatalo H, Pihlanto-Leppälä A, Korhonen H, Korpela R, et al. Alpha-lactorphin and beta-lactorphin improve arterial function in spontaneously hypertensive rats. Life Sci 2002;71:1245–53.

[21] Bravo, F.I; Arola, L.; Muguerza B. Procedure for obtaining a hydrolyzate claw chicken leg with antihypertensive activity, and peptides obtained hydrolyzate containing. Patent No. ES2606954B1. 11 December 2017

[22] Mas-Capdevila A, Pons Z, Aleixandre A, Bravo FI, Muguerza B. Dose-Related Antihypertensive Properties and the Corresponding Mechanisms of a Chicken Foot Hydrolysate in Hypertensive Rats. Nutrients 2018. doi:10.3390/nu10091295.

[23] López-Fandiño R, Martos G, Contreras P, Molina E. Egg white ovalbumin digestion mimicking physiological conditions. J Agric Food Chem 2010;58:5640–8. doi:10.1021/jf904538w.

[24] Buñag RD, Butterfield J. Tail-cuff blood pressure measurement without external preheating in awake rats. Hypertension n.d.;4:898–903.

[25] Aluko RE. Antihypertensive Peptides from Food Proteins. Annu Rev Food Sci Technol 2015;6:235–62.

[26] Li G-H, Le G-W, Shi Y-H, Shrestha S. Angiotensin I–converting enzyme inhibitory peptides derived from food proteins and their physiological and

pharmacological effects. Nutr Res 2004;24:469–86.

[27] Martínez-Maqueda D, Miralles B, Recio I, Hernández-Ledesma B. Antihypertensive peptides from food proteins: A review. Food Funct 2012;3:350–61. doi:10.1039/c2fo10192k.

[28] Li M, Zhou L, Ma G, Cao S, Dong S. The cardiovascular effects of a chimeric opioid peptide based on morphiceptin and PFRTic-NH2. Peptides 2013;39:89–94. doi:10.1016/j.peptides.2012.10.014.

[29] Ijäs H, Collin M, Finckenberg P, Pihlanto-Leppälä A, Korhonen H, Korpela R, et al. Antihypertensive opioid-like milk peptide α-lactorphin: Lack of effect on behavioural tests in mice. Int Dairy J 2004;14:201–5. doi:10.1016/j.idairyj.2003.08.002.

[30] Reid JL, Rubin PC, Petty MA. Opioid Peptides and Central Control of Blood Pressure. Clin Exp Hypertens Part A Theory Pract 1984;6:107–20. doi:10.3109/10641968409062554.

[31] Toda N, Kishioka S, Hatano Y, Toda H. Modulation of Opioid Actions by Nitric Oxide Signaling. Anesthesiology 2009;110:166–81.

[32] Iwaniak A, Minkiewicz P, Darewicz M. Food-originating ACE inhibitors, including antihypertensive peptides, as preventive food components in blood pressure reduction. Compr Rev Food Sci Food Saf 2014;13:114–34.

[33] Atkinson AB, Robertson JIS. Captopril in the Treatment of Clinical Hypertension and Cardiac Failure. Lancet 1979;314:836–9. doi:10.1016/S0140-6736(79)92186-X.

[34] Da A, Lasuncio MA, Quiro A. Bioavailability of the antihypertensive peptide LHLPLP : Transepithelial flux of HLPLP 2008;18:279–86.

TABLES

Table 1. *In silico* simulated digestion of the peptide AVFQHNCQE

Original sequence	Enzyme[a]	Digestion stage	Final sequence
AVFQHNCQE	Pepsin	Gastric	VFQHNCQE
AVFQHNCQE	Pepsin	Gastric	AV
AVFQHNCQE	Pepsin	Gastric	FQHNCQE
VFQHNCQE	Chymotrypsin	Duodenal	VF
VFQHNCQE	Chymotrypsin	Duodenal	QHNCQE
QHNCQE	Chymotrypsin	Duodenal	VFQH
QHNCQE	Chymotrypsin	Duodenal	NCQE
VFQHNCQE	Chymotrypsin	Duodenal	QHNCQE
VFQHNCQE	Chymotrypsin	Duodenal	FQHNCQE
FQHNCQE	Chymotrypsin	Duodenal	QHNCQE
FQHNCQE	Chymotrypsin	Duodenal	FQH
FQHNCQE	Chymotrypsin	Duodenal	NCQE
FQHNCQE	Chymotrypsin	Duodenal	QH
FQHNCQE	Chymotrypsin	Duodenal	NCQE

In silico simulated digestion of peptide AVFQHNCQE using the bioinformatics tool, Peptide Cutter from Expasy®. [a] Pepsin and chymotrypsin were the enzymes that hydrolyzed the peptide in gastric and duodenal stage, respectively. Trypsin, lipase and colipase were also evaluated showing no effects on peptide hydrolysis.

Table 2. Identified sequences from *in vitro* digestion of the peptide AVFQHNCQE

Peptide sequence[a]	m/z[b]	Mass monoisotopic (Da)	RT[c]	Charge	Area D0[d]	Area D60[e]
AVFQHNCQE	538.233	1,074.451	4.86	2	5,236,186	13,652
AVFQHNCQE(S-S)AVFQHNCQE	537.730	2,146.887	5.24	4	605,812	-
AVF	336.191	335.183	5.83	1	200,712	-
AVFQHNC	409.683	817.350	4.72	2	220,051	-
AVFQHN	358.179	714.343	4.24	2	685,482	-
AVFQHNCQE(S-S)QHNCQE	458.436	1,829.715	4.11	4	136,056	-
AVFQH	301.158	600.300	4.34	2	155,533	-
AVFQHNCQE(S-S)CQE	484.526	1,450.554	4.25	3	93,694	-
QHNCQE	379.647	757.279	1.23	2	288,536	335,871
NCQE	493.169	492.161	1.27	1	-	57,009

[a] Amino acid residues are designated using their one letter codes.
[b] m/z=mass-to-charge
[c] RT= retention time
[d] G60= samples from 60 min of gastric digestion
[e] D60= samples from 60 min of duodenal digestion.

Table 3. Recovery and reproducibility of the evaluated extraction methods

Method	Rec. (%, n=3)[a]	RSD (%, n=3)[b]
SPE	92.4	2.5
TFA	48.2	28.3
TFA + SPE	37.8	26.5

SPE, plasma + solid phase extraction; TFA, plasma + trifluoroacetic acid; TFA + SPE, trifluoroacetic acid + solid phase extraction. [a] Average percent recovery of the peptides by the described method. [b] Repeatability (RSD—relative standard deviations—of peak response and retention time).

Table 4. Transport of peptide and fragments from D0 through Caco-2 monolayer

Peptide sequence[a]	m/z[b]	Mass monoisotopic (Da)	RT[c]	Charge	D0 % transport[d]
AVFQHNCQE	538.233	1,074.451	4.86	2	0.25
AVFQHNCQE(S-S)AVFQHNCQE	537.730	2,146.887	5.24	4	0.11
AVF	336.191	335.183	5.83	1	0.15
AVFQHNC	409.683	817.350	4.72	2	0.60
AVFQHN	358.179	714.343	4.24	2	0.10
AVFQHNCQE(S-S)QHNCQE	458.436	1,829.715	4.11	4	0.15
AVFQH	301.158	600.300	4.34	2	0.17
AVFQHNCQE(S-S)CQE	484.526	1,450.554	4.25	3	0.15
QHNCQE	379.647	757.279	1.23	2	N.D
NCQE	493.169	492.161	1.27	1	N.D

[a] Amino acid residues are designated using their one letter codes.
[b] m/z=mass-to-charge
[c] RT= retention time
[d] % transport: percentage of the peptide or peptide fragments content found in basolateral chamber in comparison to the apical chamber content. D60= samples from 60 min of duodenal digestion.

FIGURES

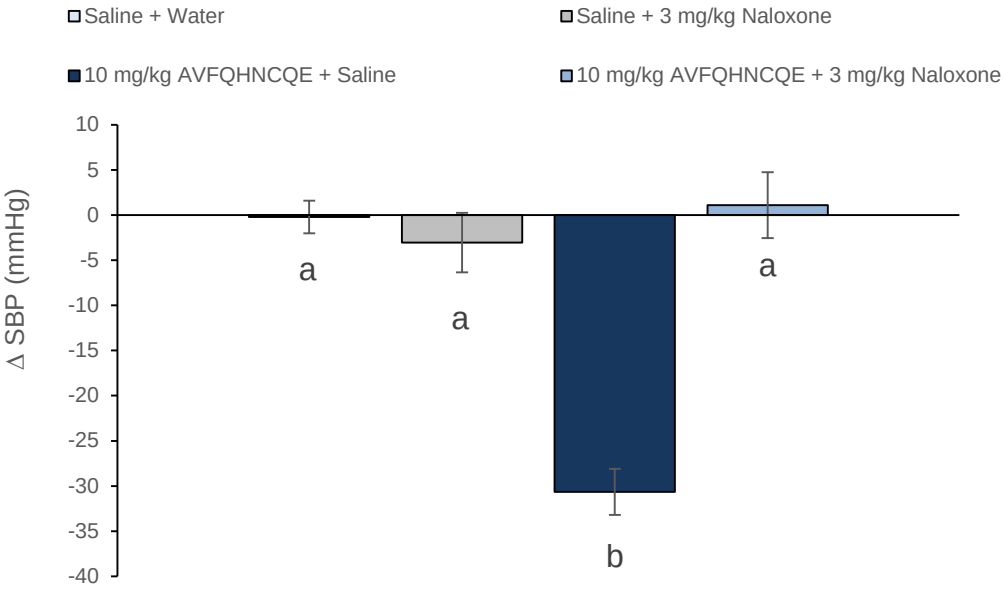

Figure 1. Changes in systolic blood pressure (SBP) caused in spontaneously hypertensive rats after 6 h post-administration of the different treatments: oral administered water + intraperitoneal injected saline, oral administered water saline + intraperitoneal injected 3 mg/kg bw Naloxone, oral administered water 10 mg/kg bw AVFQHNCQE + intraperitoneal injected saline or oral administered water 10 mg/kg bw AVFQHNCQE + intraperitoneal injected 3 mg/kg bw Naloxone. Data are expressed as the mean ± standard errors. The experimental groups always had six animals each. Different letters represent statistically significant differences ($p < 0.05$). P was estimated by one-way ANOVA.

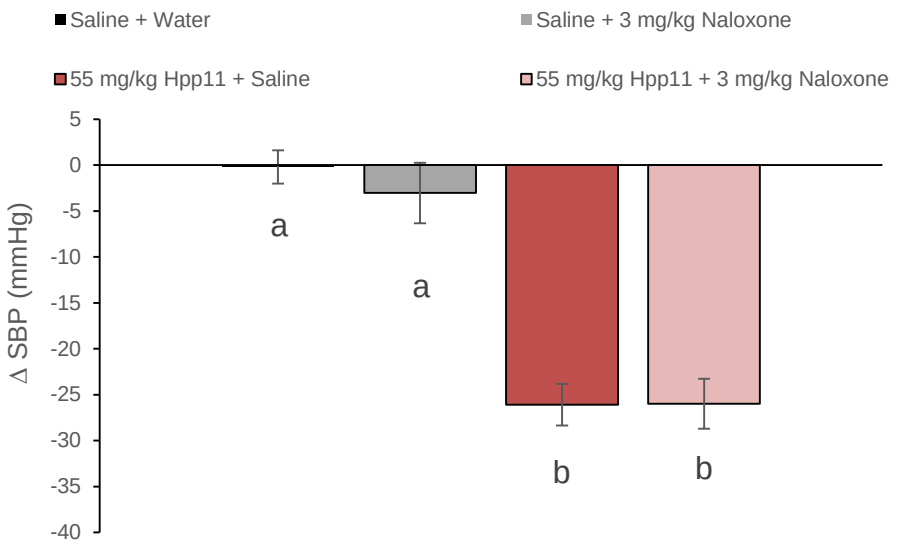

Figure 2. Changes in systolic blood pressure (SBP) caused in spontaneously hypertensive rats after 6 h post-administration of the different treatments: oral administered water + intraperitoneal injected saline, oral administered saline + intraperitoneal injected 3 mg/kg bw Naloxone, oral administered 55 mg/kg Hpp11 + intraperitoneal injected saline or oral administered 55 mg/kg bw Hpp11 + intraperitoneal injected 3 mg/kg bw Naloxone. Data are expressed as the mean ± standard errors. The experimental groups always had six animals each. Different letters represent statistically significant differences ($p < 0.05$). P was estimated by one-way ANOVA.

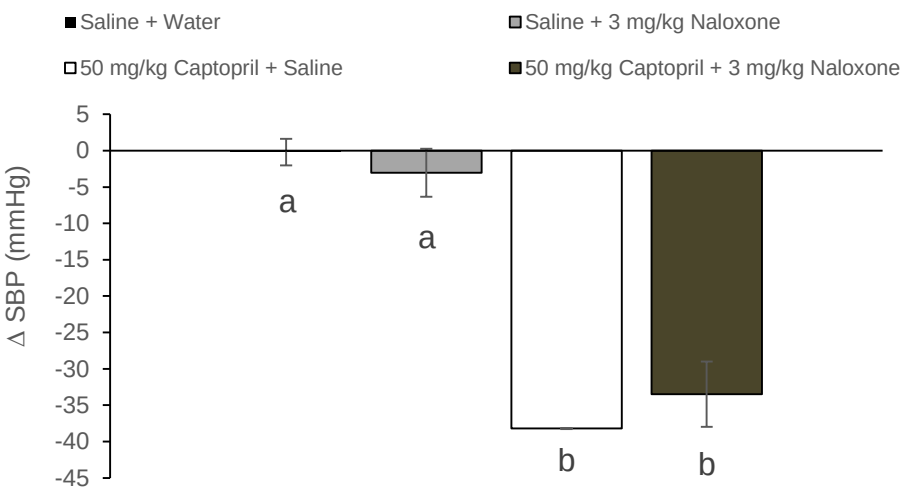

Figure 3. Changes in systolic blood pressure (SBP) caused in spontaneously hypertensive rats after 6 h post-administration of the different treatments: oral administered water + intraperitoneal injected saline, oral administered saline + intraperitoneal injected 3 mg/kg bw Naloxone, oral administered 50 mg/kg bw Captopril + intraperitoneal injected saline or oral administered 50 mg/kg Captopril + intraperitoneal injected 3 mg/kg bw Naloxone. Data are expressed as the mean ± standard errors. The experimental groups always had six animals each. Different letters represent statistically significant differences ($p < 0.05$). P was estimated by one-way ANOVA.

GENERAL DISCUSSION

GENERAL DISCUSSION

The link between the prevalence of diseases such as CVD or HTN to dietary factors has been extensively demonstrated through epidemiological evidence (1). Therefore, the use of bioactive compounds is emerging in response to the increased perception on the relationship between food and health (2). In this sense, dietary proteins have demonstrated that they could be considered a potential source of bioactive peptides with a wide range of biological activities (3). Among these bioactive peptides, those presenting antihypertensive activity have attracted great attention due to their potential beneficial effects related to HTN and CVD management (4).

HTN, affecting more than 874 million adults worldwide (5), is the most common preventable risk factor for CVD and is considered the leading single contributor to all-cause mortality and disability worldwide (6). The relationship between high BP values and increased risk of CVD is graded and continuous. Thus, a successful prevention and treatment of HTN are key to reduce disease-derived problems and to promote longevity in the world's population (7). Depending on the HTN stage, the intervention ranges from lifestyle modifications to pharmacological therapy (8). However, the use of antihypertensive drugs, especially ACE-inhibitors, could be associated with several negative side effects in some patients (9). These undesirable effects lead to reduce patient compliance with treatment, increasing health care costs and preventable fatalities. Therefore, the research focused on the development of natural protein-derived compounds that could alleviate or ameliorate HTN is receiving great attention (10).

Certain dietary proteins, comprising those from vegetal and animal origin have been reported to be a source of bioactive peptides, including those with ACEI and antihypertensive activity. Accordingly, chicken proteins have been demonstrated to be a source of ACEI peptides and also antihypertensive peptides (11–13). Considering that tones of chicken by-products are generated every year, representing economic and environmental problem for the poultry industries, the obtainment of bioactive peptides from these chicken by-products has emerged as a potential alternative use of these products allowing to their revalorization (14). Indeed, some chicken by-products including skin (12), bone (15) or blood (16) have been reported to be a source of ACEI and antihypertensive peptides. However, as far as we know, chicken feet, also a poultry by-product, has never been used before. Considering this, chicken feet, subjected to specific proteolytic treatment, could be a source of peptides presenting ACEI activity and antihypertensive activity. Thus, the objective of this Thesis was to obtain and to identify from chicken feet proteins ACEI peptides presenting antihypertensive properties and to study the bioavailability and the mechanisms involved in the possible BP-lowering effect.

Therefore, the first objective was to explore the use of chicken feet proteins to obtain hydrolysates with ACEI activity and antihypertensive activity under optimized hydrolysis conditions **[Manuscript 1]**. Thus, chicken feet processed into a powder, diluted in water and heated at 100 ºC, was subjected to a hydrolysis procedure. Prior hydrolysis, since protein solubilisation is depending on pH, different conditions of this parameter were evaluated. Then, different hydrolysis conditions, including two enzymes (Neutrase and Protamex), two temperatures (25 and 50 ºC) and different times were assayed. The ACEI activity was determined in the obtained hydrolysates as selection criteria for the best

hydrolysis conditions to obtain hydrolysates with ACEI activity higher than 90 %. It was observed that 50 ºC was the optimal temperature to obtain chicken foot protein hydrolysates presenting more than 90 % of ACE inhibition. Indeed, only the samples obtained at time 0 h of hydrolysis showed ACEI activity of less than 90 %. In fact, the pharmacology potency of these eighteen hydrolysates was demonstrated by their low IC_{50} values, similar than those reported for milk proteins currently commercialised as antihypertensive ingredients (17). However, since high *in vitro* ACEI activity does not correlate to *in vivo* antihypertensive activity (18), the evaluation of the *in vivo* antihypertensive effect in SHR is required. Hence, three hydrolysates obtained under different hydrolysis conditions and presenting the lowest IC_{50} values were administered to SHR at dose of 5 mL/kg body weight (bw). The evaluated hydrolysates showed antihypertensive effects after their administration to SHR. These results confirm the great antihypertensive potential of chicken feet proteins and could be used to formulate antihypertensive functional foods or nutraceuticals.

Posteriorly, considering the therapeutic applicability of this chicken foot hydrolysate in HTN maintenance, a dose optimisation study was performed using the antihypertensive chicken foot hydrolysate 11 (Hpp11), obtained after 2 h 50 ºC hydrolysis with protamex [**Manuscript 2**]. Three different low doses 85, 55 and 25 mg/kg bw were administered to SHR to identify the dose with the maximum antihypertensive effect. Administered doses, 85 and 55 mg/kg, showed similar antihypertensive effect. Remarkably, both doses exhibited a BP decrease similar to that caused by the antihypertensive drug Captopril, confirming the great antihypertensive potential of Hpp11. Moreover, the dose of 55 mg/kg maintained the antihypertensive effect 8 h post-administration. On the other hand, a possible undesirable hypotensive effect of chicken foot

hydrolysate Hpp11 was rule out when administered to the normotensive control of SHR, WKY rats, linking Hpp11 effect to pathogenic state. Furthermore, it was demonstrated that Hpp11 competitively binds to ACE, a key enzyme in BP control. Other ACE inhibitory peptides, competitively block the binding of Ang I to ACE, thereby inhibiting the formation of Ang II and thus, reducing the vasoconstriction (19). In addition, Hpp11 reduced ACE activity in plasma, indicating that this can be the mechanism involved in Hpp11 antihypertensive effect. Nevertheless, it is known that antihypertensive peptides can reduce BP through other mechanisms than ACE inhibition. In this sense, the effect of Hpp11 on vascular reactivity was also studied in pre-contracted aortic rings preparations. However, Hpp11 did not produce vasorelaxation effect in aortic ring preparations. Therefore, these results confirmed that the optimal dose of chicken foot hydrolysate was 55 mg/kg, showing similar antihypertensive effect than this produced by Captopril and link the effect of this hydrolysate to the presence of HTN.

Nevertheless, considering that HTN is a chronic disease requiring chronic treatment (20), we aimed to evaluate the antihypertensive effect of the long-term administration of Hpp11 in cafeteria-diet-fed rats **[Manuscript 3]**. This dietary model is an exceptional tool to study MetS (21) including diet-induced HTN (22). Agreeing to previous reported, in this study, after 8 weeks of CAF diet rats developed diet-induced HTN. Since 9th week and for 3 weeks rats fed CAF diet received a daily oral dose of Hpp11 (55 mg/kg bw) which was able to decrease BP since the first week of administration. Contrary, the untreated rats increased BP as CAF diet weeks progressed. Remarkably, at the end of the experiment, Hpp11 produced an important decrease in BP of -20 mmHg compared to the untreated group. These results confirm the potent antihypertensive effect of Hpp11 and their benefits in CVD, considering

that, a reduction of 10-12 mmHg in SBP may reduce the risk of stroke by 40 %, coronary heart disease by 16 % and all-cause mortality by 13 % (23). Other protein hydrolysates reported antihypertensive effect after long-term administration in SHR (24,25) nevertheless, to our knowledge, this is the first study demonstrating the antihypertensive effect of a protein hydrolysate in CAF-diet fed rats. These results are relevant considering that CAF-diet fed rats, such as most HTN patients, present besides of HTN, the associated cardiometabolic risk factors. Thus, Hpp11 antihypertensive effect in this animal model open doors to the use of this hydrolysate in diet-induced HTN management.

Interestingly, long-term administration of Hpp11 not only induced antihypertensive effect but also demonstrated to exert antioxidant effect by enhancing the production of GSH, a key antioxidant system that reduces oxidative stress by removing ROS (26). The antioxidant effects of certain proteins were already known (27,28). Indeed, antioxidant effect of Hpp11 could be involved in the observed BP reduction considering that no changes were observed in plasmatic ACE activity. Additionally, it seems that the reduction in mRNA expression of the vasoconstrictor ET-1 and the increase in mRNA expression of Sirt-1, enhancer of NO availability, could contribute to the beneficial effect of Hpp11 in diet-induced hypertensive rats improving endothelium functionality. In addition, mRNA expression of NOX4 was found increased in CAF-diet rats administered Hpp11. This upregulation of NOX4 was linked to NOX4 vasoprotective effects considering that healthy rats fed STD diet showed similar mRNA expression levels of this parameter. NOX4 overexpression could contribute to improve the endothelium through enhancing NO bioavailability (29). Therefore, these results demonstrated that Hpp11 administered during 3 weeks reduced

BP through exerting antioxidant effect and conferring endothelial vasoprotective pattern in diet-induced hypertensive rats.

Considering the previous result, it was demonstrated that the hydrolysis of chicken foot proteins under optimised hydrolysis conditions resulted in the obtainment of antihypertensive hydrolysates including the Hpp11. In the following study we aimed to identify those antihypertensive peptides contained in chicken foot hydrolysate Hpp11 [**Manuscript 4**]. Thus, Hpp11 was subjected to peptide separation according to molecular size and hydrophobicity by ultrafiltration and RP-HPLC. The posterior selection according to the exerted ACEI allowed to identify the hydrolysate fractions presenting the highest ACEI activity. Then, peptide identification by HPLC-MS in the fractions showing the high ACEI activity resulted in the obtainment of 1020 identified peptides. According to their intensity, size and/or sequence eight peptides were selected to be synthetized and five of them showed high ACEI activity, lower than 100 μM. Interestingly, two of the identified peptides, the sequences AVFQHNCQE and QVGPLIGRYCG, also showed antihypertensive effect when administered at low dose of 10 mg/kg bw to SHR, suggesting that those peptides could be involved in Hpp11 antihypertensive effect. Interestingly, the antihypertensive effect of peptides derived from other chicken proteins was previously reported, however the administered doses were very variable, ranging from 10 mg/kg to 60 mg/kg to obtain similar BP decrease (15,30). Although other identified peptides in Hpp11 showed high ACEI activity than the peptide AVFQHNCQE, this peptide was also demonstrated to present antihypertensive effect. These findings demonstrate that high *in vitro* ACE inhibitory activity does not always correlates to potent antihypertensive effect.

In order to evaluate the participation of endothelial-related relaxing factors the peptide antihypertensive effect different experiments were carried out. In this sense, to evaluate if peptide antihypertensive effect was dependent of the vasodilators NO or PGI_2, AVFQHNCQE antihypertensive effect was studied in SHR also peritoneal injected L-NAME, inhibitor of NO synthesis, or indomethacin, inhibitor of COX, the main responsible of PGI_2 synthesis. It was demonstrated that AVFQHNCQE produced NO-mediated antihypertensive considering that in presence of L-NAME, the peptide antihypertensive effect disappeared. This result demonstrated that the vasodilation of the vessels induced by NO could be the mechanism underlying the antihypertensive effect of the peptide [**Manuscript 5**]. According with that, when it was evaluated the plasmatic ACE activity in the group administered the peptide no significant reduction was found, showing similar ACE activity values than the group that received water demonstrating that ACE inhibition is not involved in peptide BP-lowering effect. NO-mediated antihypertensive effect was previously reported for antihypertensive peptides (31), especially in those peptides showing opioid properties (32) which do not require to be absorbed to produce their effects (33). Indeed, in a posterior study evaluating the peptide bioavailability in plasma and its transepithelial transport through Caco-2 monolayer it was observed that AVFQHNCQE was not absorbed through intestinal epithelium [**Manuscript 6**]. Therefore, the implication of the opioid receptors in the peptide antihypertensive effect was studied in presence of Naloxone, an opioid receptor antagonist. The results showed that in the animals receiving Naloxone, the AVFQHNCQE BP-lowering effect disappeared, evidencing that the interaction with opioid receptors was crucial to exert an antihypertensive effect. In fact, opioid receptors have been described in

the GIT (34) and it is extensively demonstrated that their activation could interfere in BP regulation (35). Thus, the stimulation of opioid receptors by AVFQHNCQE in GIT, without requiring its absorption, could enhance NO bioavailability and thus, induce vasodilation and consequently reduce BP.

To evaluate the molecular mechanisms involved in the NO-mediated antihypertensive effect of AVFQHNCQE, the implication of endothelial genes related to NO-production pathway were evaluated. Nevertheless, no significant changes were observed neither in mRNA expression of NO producer (eNOS) nor in mRNA expression of NO inducers (Sirt-1, KLF-2 and Arg-1). Hence, only a decrease in vasocontrictior ET-1 and in NOX4 was observed leading to an increase NO availability considering that ET-1 also inhibits NO production and NOX4 in SHR is clearly related with an increase in ROS production (36) [**Manuscript 5**]. Therefore, an improvement in endothelial function by reducing ET-1 and NOX4 expression and thus, increasing NO bioavailability, could be the mechanisms underlying the NO-mediated antihypertensive effect of AVFQHNCQE.

Considering all the results of this Thesis, it was demonstrated that the hydrolysis of chicken foot proteins under the optimal hydrolysis conditions resulted in the release of antihypertensive peptides. Chicken foot protein hydrolysate Hpp11 reduced BP after acute administration of a low dose of 55 mg/kg. As expected, considering that Hpp11 was selected according to their *in vitro* ACEI activity, it was demonstrated that *in vivo* reduction of plasmatic ACE and the exerted antioxidant effect are the main mechanisms involved in Hpp11 acute antihypertensive effect. Furthermore, it was demonstrated that its effect was linked to the presence of pathogenic state, being not observed in

WKY. In addition, long-term administration of Hpp11 presented antihypertensive effect in diet model of HTN conferring a vasoprotective endothelial pattern. The long-term antihypertensive effect produced by Hpp11, reaching a decrement of -18 mmHg when compared to untreated group could be directly associated with a reduction in major CVD events (37). Therefore, Hpp11 could be a potential alternative in HTN treatment. The posterior analysis by HPLC-MS in the Hpp11 most active fractions allowed the identification of antihypertensive peptides, including the peptide AVFQHNCQE.

It was demonstrated that the NO-mediated antihypertensive AVFQHNCQE produced after acute administration when administered to SHR, and this effect is mediated through their interaction with opioid receptors in GIT. Opioid peptides do not require its absorption to produce their effect (33), this could explain the antihypertensive effect of AVFQHNCQE even demonstrated that it was not absorbed. NO-mediated antihypertensive effect could be explained by the antioxidant effect produced by the peptide, reducing NO scavenging and also, by an improvement in the endothelial function. The reduction in ET-1 and NOX4 expression could contribute to the NO increase considering that ET-1 inhibits NO synthesis and NOX4 is known as ROS producer in SHR. The present findings clearly indicate that in SHR, AVFQHNCQE activates an opioid-mediated, cardiovascular depressor mechanism; however, further studies are needed to elucidate which type of opioid receptors are activated by AVFQHNCQE and how this opioid-mediated activity enhances NO availability and thus, produce the BP reduction.

REFERENCES

1. Stewart J, Manmathan G, Wilkinson P. Primary prevention of cardiovascular disease: A review of contemporary guidance and literature. JRSM Cardiovasc Dis [Internet]. SAGE Publications; 2017;6:2048004016687211.

2. Scientific Concepts of Functional Foods in Europe Consensus Document PREFACE ILSI Europe's role.

3. Korhonen H, Pihlanto A. Food-derived Bioactive Peptides – Opportunities for Designing Future Foods. 2003;1297–308.

4. Gu RZ, Liu WY, Lin F, Jin ZT, Chen L, Yi WX, et al. Antioxidant and angiotensin I-converting enzyme inhibitory properties of oligopeptides derived from black-bone silky fowl (Gallus gallus domesticus Brisson) muscle. Food Res Int. Elsevier Ltd; 2012;49(1):326–33.

5. Forouzanfar MH, Liu P, Roth GA, Ng M, Biryukov S, Marczak L, et al. Global Burden of Hypertension and Systolic Blood Pressure of at Least 110 to 115 mm Hg, 1990-2015. JAMA. 2017 Jan 10;317(2):165.

6. Forouzanfar MH, Afshin A, Alexander LT, Anderson HR, Bhutta ZA, Biryukov S, et al. Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet. 2016 Oct 8 [;388(10053):1659–724.

7. 2017 Guideline for High Blood Pressure in Adults - American College of Cardiology [Internet]. Available from: https://www.acc.org/latest-in-cardiology/ten-points-to-remember/2017/11/09/11/41/2017-guideline-for-high-blood-pressure-in-adults

8. Cuspidi C, Tadic M, Grassi G, Mancia G. Treatment of hypertension: The ESH/ESC guidelines recommendations. Pharmacol Res. Elsevier Ltd; 2018;128:315–21.

9. Sica DA. Angiotensin-converting enzyme inhibitors side effects--physiologic and non-physiologic considerations. J Clin Hypertens (Greenwich). 2004;6(7):410–6.

10. Möller NP, Scholz-Ahrens KE, Roos N, Schrezenmeir J. Bioactive peptides and proteins from foods: Indication for health effects. Eur J Nutr. 2008;47(4):171–82.

11. Bravo, F.I; Arola, L.; Muguerza B. Procedure for obtaining a hydrolyzate claw chicken leg with antihypertensive activity, and peptides obtained hydrolyzate containing. Spain: ES2606954B1; 2017.

12. Onuh JO, Girgih AT, Malomo SA, Aluko RE, Aliani M. Kinetics of in vitro renin and angiotensin converting enzyme inhibition by chicken skin protein hydrolysates and their blood pressure lowering effects in spontaneously hypertensive rats. J Funct Foods. Elsevier Ltd; 2015;14:133–43.

13. Yoshii H, Tachi N, Ohba R, Sakamura O, Takeyama H, Itani T. Antihypertensive effect of ACE inhibitory oligopeptides from chicken egg yolks. Comp Biochem Physiol - C Pharmacol Toxicol Endocrinol. 2001;128(1):27–33.

14. Williams CM. Poultry waste management in developing countries. Available from: http://www.fao.org/docrep/019/i3531e/i3531e05.pdf

15. Nakade K, Kamishima R, Inoue Y, Ahhmed A, Kawahara S, Nakayama T, et al. Identification of an antihypertensive peptide derived from chicken bone extract. Anim Sci J. 2008;79(6):710–5.

16. Huang S, Liu P. Inhibition of Angiotensin I - Converting Enzymes by Enzymatic Hydroiysates from Chicken Blood. J Food Drug Anal. 2010;18(6):458–63.

17. Muguerza B, Ramos M, Sánchez E, Manso M a., Miguel M, Aleixandre a., et al. Antihypertensive activity of milk fermented by Enterococcus faecalis strains isolated from raw milk. Int Dairy J. 2006;16(1):61–9.

18. Martin M, Deussen A. Effects of natural peptides from food proteins on angiotensin converting enzyme activity and hypertension. Crit Rev Food Sci Nutr. Taylor & Francis; 2017;8398(December):1–20.

19. Ahhmed AM, Muguruma M. A review of meat protein hydrolysates and hypertension. Meat Sci. 2010;86(1):110–8.

20. Arima H, Barzi F, Chalmers J. Mortality patterns in hypertension. J Hypertens [Internet]. 2011 Dec;29:S3–7.

21. Sampey BP, Vanhoose AM, Winfield HM, Freemerman AJ, Muehlbauer MJ, Fueger PT, et al. Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation : Comparison to high-fat diet. Obesity. Nature Publishing Group; 2009;19(6):1109–17.

22. Pons Z, Margalef M, Bravo FI, Arola-arnal A, Muguerza B. Chronic administration of grape-seed polyphenols attenuates the development of hypertension and improves other cardiometabolic risk factors associated with the metabolic syndrome in cafeteria diet-fed rats. 2018;(2017):200–8.

23. Jäkälä P, Vapaatalo H. Antihypertensive peptides from milk proteins. Pharmaceuticals. 2010;3(1):251–72.

24. Manso MA, Miguel M, Even J, Hernández R, Aleixandre A, López-Fandiño R. Effect of the long-term intake of an egg white hydrolysate on the oxidative status and blood lipid profile of spontaneously hypertensive rats. Food Chem. 2008;109(2):361–7.

25. Sánchez D, Kassan M, Contreras MDM, Carrón R, Recio I, Montero MJ, et al. Long-term intake of a milk casein hydrolysate attenuates the development of hypertension and involves cardiovascular benefits. Pharmacol Res. 2011;63(5):398–404.

26. Quiñones M, Miguel M, Muguerza B, Aleixandre A. Effect of a cocoa polyphenol extract in spontaneously hypertensive rats. Food Funct. 2011;2(11):649.

27. Liu R, Wang L, Zheng W, Wu H. In vivo antioxidant effects of hydrolysate derived from waste proteins of Mactra veneriformis. J Aquat Food Prod Technol. Taylor & Francis; 2015;24(2):143–52.

28. Elias RJ, Kellerby SS, Decker EA. Antioxidant activity of proteins and peptides. Crit Rev Food Sci Nutr. 2008;48(5):430–41.

29. Drummond GR, Sobey CG. Endothelial NADPH oxidases: which NOX to target in vascular disease? Trends Endocrinol Metab. Elsevier; 2014 Sep 1;25(9):452–63.

30. Fujita, Yokoyama, Yoshikawa M, Iroyukifujita H, Eiichiyokoyama K. Classification

and Antihypertensive Activity of Angiotensin I-Converting Enzyme Inhibitory Peptides Derived from Food Proteins. J Food Sci. 2000;65(4):564–9.

31. Marques C, Manuela Amorim M, Odila Pereira J, Estevez Pintado M, Moura D, Calhau C, et al. Bioactive Peptides - Are There More Antihypertensive Mechanisms Beyond ACE Inhibition? Curr Pharm Des. 2012;18(30):4706–13.

32. Majumder K, Wu J. Molecular Targets of Antihypertensive Peptides : Understanding the Mechanisms of Action Based on the Pathophysiology of Hypertension. 2015;256–83.

33. Miner-Williams WM, Stevens BR, Moughan PJ. Are intact peptides absorbed from the healthy gut in the adult human? Nutr Res Rev. 2014;27(2):308–29.

34. Holzer P. Opioid receptors in the gastrointestinal tract. Regul Pept. 2009;155(1–3):11–7.

35. Reid JL, Rubin PC, Petty MA. Opioid Peptides and Central Control of Blood Pressure. Clin Exp Hypertens Part A Theory Pract. 1984;6(1–2):107–20.

36. Konior A, Schramm A, Czesnikiewicz-Guzik M, Guzik TJ. NADPH Oxidases in Vascular Pathology. Antioxid Redox Signal. 2014 Jun 10;20(17):2794–814.

37. Ettehad D, Emdin CA, Kiran A, Anderson SG, Callender T, Emberson J, et al. Blood pressure lowering for prevention of cardiovascular disease and death: A systematic review and meta-analysis. Lancet. Elsevier Ltd; 2016;387(10022):957–67.

CONCLUSIONS

CONCLUSIONS

1. The hydrolysis of chicken foot proteins under optimized conditions (2 h, 50 °C, pH=7 hydrolysis with Protamex) results in the obtaining of hydrolysates with antihypertensive properties.
2. The most effective dose of chicken foot hydrolysate Hpp11 was 55 mg/kg bw showing the maximum antihypertensive effect 6 h post-administration to SHR. The BP decrease pattern observed in rats administered peptide was similar than the observed in the group administered the antihypertensive drug Captopril.
3. Chicken foot hydrolysate Hpp11 (55 mg/kg bw) did not reduced BP in WKY, the normotensive control of the SHR. Therefore, Hpp11 effect is linked to hypertension condition.
4. The reduction of ACE activity was the mechanism underlying the chicken foot hydrolysate Hpp11 antihypertensive effect after its acute administration.
5. Long-term administration of chicken foot hydrolysate Hpp11 (55 mg/kg bw) significantly reduced BP in diet-induced hypertensive rats.
6. Chicken foot hydrolysate Hpp11 reduced BP in diet-induced hypertensive rats through exhibiting antioxidant effect and improving endothelial function, enhancing the expression of Sirt-1 and NOX4 and decreasing the expression of ET-1.
7. AVFQHNCQE and QVGPLIGRYCG, were the antihypertensive peptides identified in chicken foot hydrolysate Hpp11.
8. AVFQHNCQE produced a NO-mediated antihypertensive effect. AVFQHNCQE contributed to enhance NO availability by

exhibiting antioxidant effect and reducing the expression of NO scavengers, such as ET-1 and NOX4.

9. AVFQHNCQE produced its antihypertensive effect through the interaction with opioid receptors and therefore, its absorption through the intestinal epithelium was not required.

The hydrolysis of chicken foot proteins under optimized conditions is a good strategy for the obtainment of antihypertensive hydrolysates and peptides. One of the obtained hydrolysates, the Hpp11, reduced BP after its acute and chronic administration by reducing ACE activity and by improving endothelial function. The antihypertensive peptides identified in Hpp11, QVGPLIGRYCG and AVFQHNCQE, which was demonstrated to exert its effect through the interaction with opioid receptors in GIT, could contribute to Hpp11 antihypertensive effect.

Thus, the obtained antihypertensive hydrolysate and peptides could be good candidates to be used as nutraceuticals or to be included in a functional food for the mitigation and the prevention of HTN.

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GRÀCIES

ANNEX

LIST OF PUBLICATIONS

A) Published papers:

Mas-Capdevila, A., Pons, Z., Aleixandre, A., Bravo, F.I., Muguerza, B. 2018. Dose-related antihypertensive properties and the corresponding mechanisms of a chicken feet hydrolysate in hypertensive rats. Nutrients. 10: 1295. Impact factor (2017): 4.19. SI Journal Citation Reports © Ranking: 18/83 (Q1) (Nutrition and dietetics)

Mas-Capdevila, A., Iglesias-Carres, L., Pons, Z., Arola-Arnal, A., Bravo, F.I., Muguerza, B. 2018. Relationship between dose and antihypertensive effect for an ACE inhibitor chicken feet hydrolysate. Annals of Nutrition and Metabolism 73 (2): 60. Impact factor (2017): 3.05. SI Journal Citation Reports © Ranking: 36/83 (Q2) (Nutrition and dietetics)

Iglesias-Carres, L., **Mas-Capdevila, A.,** Bravo, F.I., Arola-Arnal, A., Muguerza, B. 2018. Differences in the serum kinetics behavior of organic and conventional whole red grape phenolic metabolites. Annals of Nutrition and Metabolism 73 (2): 52. Impact factor (2017): 3.05. SI Journal Citation Reports © Ranking: 36/83 (Q2) (Nutrition and dietetics)

B) Submitted papers:

Bravo, F.I., **Mas-Capdevila, A.**, Margalef, M., Arola-Arnal, A., Muguerza, B. Antihypertensive effect of hydrolysates from chicken feet proteins. [Submitted to Food Funct.]

Mas-Capdevila, A., Iglesias-Carres, L., Arola-Arnal, A., Suarez, M., Muguerza, B., Bravo, F.I.. Long-term administration of the chicken feet hydrolysate Hpp11 decreases blood pressure and confers a vasoprotective pattern in diet-induced hypertensive rats. [Submitted to J Funct Foods.].

Bravo, F.I., **Mas-Capdevila, A.**, Margalef, M., Arola-Arnal, A., Muguerza, B. Novel antihypertensive peptides derived from chicken foot proteins . [Submitted to Mol. Nutr. Food Res].

Mas-Capdevila, A., Iglesias-Carres, L., Arola-Arnal, A., Aragonès G., Aleixandre, A., Bravo, F.I., Muguerza, B. Evidence that nitric oxide is involved in the blood pressure lowering effect of AVFQHNCQE in spontaneously hypertensive rats [Submitted to Food Chem.].

Iglesias-Carres, L., **Mas-Capdevila, A.**, Bravo, F.I., Bladé, C., Arola-Arnal, A., Muguerza, B. Optimization of extraction method for characterization of phenolic compounds from apricot fruit (*Prunus armeniaca*). [Submitted to J. Food Comp. Anal.].

Iglesias-Carres, L., **Mas-Capdevila, A.**, Bravo, F.I., Aragonès, G., Muguerza, B, Arola-Arnal, A., Optimization of a polyphenol extraction method for sweet orange pulp (*Citrus sinensis L.*) to identify phenolic compounds consumed from sweet oranges. [Submitted to PLoS One.].

Iglesias-Carres, L., **Mas-Capdevila, A.,** Bravo, F.I., Muelro, M., Muguerza, B., Arola-Arnal, A. Specific extraction optimization by response surface methodology and full characterization of Royal Dawn sweet cherry (*Prunus avium*) phenolics reveal that this variety is rich in rutin. [Submitted to Food Chem.].

Iglesias-Carres, L., **Mas-Capdevila, A.**, Sancho-Pardo, Lucía., Bravo, F.I., Muelro, M., Muguerza, B., Arola-Arnal, A. Optimized extraction by response surface methodology, characterization and quantification of phenolic compounds in whole red grapes (Vitis vinifera). [Submitted to J. Agric. Food Chem.].

C) <u>In preparation papers:</u>

Mas-Capdevila, A., Iglesias-Carres, L., Arola-Arnal, A., Bladé, C., Bravo, F.I., Muguerza, B. Opioid-like activity mediates the antihypertensive effect of the bioactive peptide AVFQHNCQE and clarifies its lack of absorption.

Mas-Capdevila, A., Colom M., Guirro M., Arola-Arnal, A., Bravo, F.I., Muguerza, B. MIX of natural compounds in the management of metabolic syndrome: Hpp11 responsible of the MIX antihypertensive effect.

Mas-Capdevila, A., Iglesias-Carres, L., Laza R., Arola-Arnal, A., Bravo, F.I., Muguerza, B. Chronic supplementation with physiologic dose of grape seed proanthocyanidin reduces blood pressure in a metabolic syndrome animal model.

Mas-Capdevila, A., Iglesias-Carres, L., Arola-Arnal, A., Bravo, F.I., Muguerza, B. Chronic adminsitration of gallic acid reduces blood pressure in cafeteria-diet fed rats.

Pons, Z., Margalef M., **Mas-Capdevila, A**, Bravo, F.I., Arola-Arnal, A., Muguerza, B. Low dose of gallic acid exhibit an antihypertensive effect and confer a vasoprotective expression pattern in rats.

Iglesias-Carres, L., **Mas-Capdevila**, **A.** , Bravo, F.I., Arola-Arnal, A., Muguerza, B. Organic red grapes have a particular phenolic bioavailability in rats.

Iglesias-Carres, L., **Mas-Capdevila**, **A.** , Bravo, F.I., Muguerza, B., Arola-Arnal, A. The bioavailability and metabolism of ornge phenolic compounds is conditioned by orange growing region and photoperiod exposure.

Iglesias-Carres, L., **Mas-Capdevila, A.,** Bravo, F.I., Arola-Arnal, A., Muguerza, B. The bioavailability of red grape polyphenols is higher in winter-like conditions.

LIST OF CONFERENCE PAPERS

A) Poster communications

Bravo, F.I.; **Mas-Capdevila, A.**; Margalef, M.; Arola-Arnal, A.; Muguerza, B. Antihypertensive activity of hydrolysates obtained from chicken feet. The 2nd Conference on Food Bioactives & Health. Lisbon, 2018.

Iglesias-Carres, L., **Mas-Capdevila, A**., Bravo, F.I., Arola-Arnal, A., Muguerza, B. Administration of pure epicatechin or as part of a flavanol-rich extract does not influence flavanol metabolism and body distribution. The 2nd Conference on Food Bioactives & Health. Lisbon, 2018.

Mas-Capdevila ,A., Iglesias-Carres, L., Pons, Z., Arola-Arnal, A., Bravo, F.I., Muguerza, B. Relationship between dose and antihypertensive effect of an ACE inhibitor chicken feet hydrolysate. XVII Congreso de la Sociedad Española de Nutrición. Barcelona, 2018.

Iglesias-Carres, L., **Mas-Capdevila, A**., Bravo, F.I., Muguerza, B., Arola-Arnal, A. Differences in the serum kinetic behavior of organic and conventional whole red grape phenolic metabolites. XVII Congreso de la Sociedad Española de Nutrición. Barcelona, 2018.

Mas-Capdevila, A., Iglesias-Carres, L., Laza, R.,Arola-Arnal, A., Muguerza, B., Bravo, F.I.. Antihypertensive effect of 3-weeks supplementation with chicken-feet hydrolysate (Hpp11) in an animal model of metabolic syndrome. X Seminario sobre alimentación y estilos de vida saludables. Palma de Mallorca, 2018.

Iglesias-Carres, L., **Mas-Capdevila, A.,** Bravo, F.I., Muguerza, B., Arola-Arnal, A. Photoperiod exposure modulates sweet orange polyphenol bioavalability and metabolism. X Seminario sobre alimentación y estilos de vida saludables. Palma de Mallorca, 2018.

Mas-Capdevila, A., Laza, R., Muguerza, B, Bravo, F.I. Chronic administration of grape seed proanthocyanidin extract (GSPE) reduces blood pression in diet-induced hypertension. XL SEEBM Congress. The annual congress of SFBMM. Barcelona, 2017.

Iglesias-Carres, L., **Mas-Capdevila, A.,** Bravo, F.I., Muguerza, B., Arola-Arnal, A. Sexual hormones may explain the gender-related differences in grape seed flavanol metabolism and distribution. 8th International conference on polyphenols. Quebec, 2017.

Bravo, F.I.; **Mas-Capdevila, A.**; Laza, R.; Muguerza, B. Long-term adminsitration of Gallic Acid decrease blood pressure in metabolic syndrome animal model via an improvement of endothelial function. NuGO week 2017, Varna, Bulgaria, 2017.

Mas-Capdevila, A., Laza, R., Iglesias-Carres, L., Arola-Arnal, A., Bravo, F.I., Muguerza, B. Ácido Gálico, posible ingrediente funcional en el control de la hipertensión. IX Seminario sobre alimentación y estilos de vida saludables. Tarragona, 2017.

Iglesias-Carres, L., Sancho-Pardo, L., **Mas-Capdevila, A.**, Bravo, F.I., Muguerza, B., Arola-Arnal, A. Optimización de un método específico para extraer los compuestos fenólicos procedented del albaricoque (*Prunus armeniaca*). IX Seminario sobre alimentación y estilos de vida saludables. Tarragona, 2017.

Mas-Capdevila A., Muguerza, B., Bravo F.I. Long-term intake of Gallic Acid attenuates the development of hypertension in cafeteria diet-fed rats The 1st International Conference on Food Bioactives & Healt. Norwich, 2016.

B) Oral communications

Mas-Capdevila ,A., Iglesias-Carres, L., Arola-Arnal, A., Bravo, F.I., Muguerza, B. La administración prolongada de proantocianidinas de pepita de uva presenta efecto antihipertensivo en animales con hipertensión inducida por la

dieta. II Congreso nacional de biotecnología del vino INVINOTEC. Tarragona, 2018.

Mas-Capdevila ,A., Iglesias-Carres, L.**,** Pons, Z., Arola-Arnal, A., Bravo, F.I., Muguerza, B. Relationship between dose and antihypertensive effect of an ACE inhibitor chicken feet hydrolysate. V Reunión de Jóvenes Investigadores. Santa Coloma de Gramanet, 2018.

Iglesias-Carres, L., **Mas-Capdevila, A.**, Bravo, F.I., Muguerza, B., Arola-Arnal, A. Differences in the serum kinetic behavior of organic and conventional whole red grape phenolic metabolites. V Reunión de Jóvenes Investigadores. Santa Coloma de Gramanet, 2018.

Mas-Capdevila, A. Natural products and metabolic syndrome: Gallic acid in the management of diet-derived hypertension. Internal Medicine Research Symposium. Rotterdam, 2018.

Hypertension is considered one of the most important public health problems in our society. The treatment of this pathology is based on lifestyle modifications and pharmacology treatment. However, for those patients developing hypertension, whose blood pressure is not high enough to warrant pharmacology treatment, the use nutraceuticals or functional foods with antihypertensive properties have attracted considerable interest as good strategy to avoid the development of hypertension.

In this regard, this thesis aims to obtain antihypertensive peptides through the hydrolysis of chicken foot proteins, a by-product from poultry industries. Thus, through the hydrolysis of chicken foot proteins, it was obtained an hydrolysate, Hpp11, exerting antihypertensive effect after acute and chronic administration. Hpp11 administered acutely produced antihypertensive effect by reducing the activity of angiotensin converting enzyme, while when administered chronically the antihypertensive effect was mediated by an improvement in the endothelial function. Additionally the peptides contained in Hpp11 were characterised and two of them, AVFQHNCQE and QVGPLIGRYCG, showed antihypertensive effect. In particular, the peptide AVFQHNCQE was not absorbed and produced its antihypertensive effect through the interaction with opioid receptors from the gastrointestinal tract. The interaction with those receptors leaded to a nitric oxide-mediated antihypertensive effect. Moreover, the peptide contributed to enhance nitric oxide by exhibiting antioxidant effect and improving endothelial function. The results of this thesis open the doors to the use of the antihypertensive hydrolysate and peptides in functional foods or nutraceuticals for the control and prevention of hypertension.