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Unraveling the mechanisms of action of lactoferrin-derived antihypertensive peptides: ACE inhibition and beyond.

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Summary

Hypertension is one of the most important causes of cardiovascular and renal morbidity and mortality, and it represents a serious health problem in Western countries. Over the last few decades scientific interest on food-derived antihypertensive peptides has grown as an alternative to drugs in the control of systemic blood pressure. Most of these peptides target the angiotensin I-converting enzyme (ACE) but emerging evidence points to other antihypertensive mechanisms beyond ACE inhibition. The milk protein lactoferrin (LF) is a good source of orally active antihypertensive peptides the characterization of which, including *ex vivo* functional assays and *in vivo* approaches, shows that they might act on several molecular targets. This review summarizes the mechanisms of action underlying the blood pressure-lowering effects of LF-derived peptides, focusing on their interaction with different components of the renin-angiotensin (RAS) and endothelin (ET) systems. The ability of LF-derived peptides to modify the expression of genes encoding proteins involved in the nitric oxide (NO) pathway and prostaglandin synthesis is also described.

1. Introduction

During the last two decades, it has been recognized that apart from their basic nutritional role, many dietary proteins contain within their primary structure different peptide sequences that exert beneficial effects upon human health once released by food processing or by digestive enzymes during gastrointestinal transit. These bioactive peptides range in size from 2 to 50 amino acid residues and exhibit different activities, based on their inherent amino acid composition and sequence.[1-3] Moreover, some of these peptides are multifunctional, which is very attractive for dietary approaches and for functional food development.

Due to the prevalence and importance of hypertension in the Western population, scientific interest on antihypertensive peptides has grown as an alternative to drugs in the control of systemic blood pressure (BP) and prevention of associated cardiovascular disease (CVD) events.[4-6] The main target for antihypertensive food-derived peptides is the angiotensin I-converting enzyme (ACE), which *in vitro* inhibition is well established. However, the *in vivo* mechanism underlying vasoactive and BP-lowering effects of antihypertensive food-derived peptides has not yet been fully established and emerging evidence points to other antihypertensive mechanisms beyond ACE inhibition.[7-9]

The milk protein lactoferrin (LF), which possesses a diverse range of physiological functions such as antimicrobial/antiviral, immunomodulatory and antioxidant activities,[10-12] was a decade ago pointed out by *in silico* studies as a promising source of ACE-inhibitory peptides.[13] Since then, our group focused on the characterization of the antihypertensive effects of LF-derived peptides using different experimental approaches and models. In this review, first we briefly describe the importance of hypertension, the physiological control of BP and the

potential role of milk protein-derived peptides in hypertension management. Then the paper focuses on the scientific knowledge about the BP-lowering effects of LF-derived peptides and hydrolysates and the mechanisms underlying their antihypertensive effects, which seem not only limited to ACE inhibition.

2. Hypertension

Hypertension, or raised BP, is defined as a systolic BP (SBP) equal to or above 140 mm Hg and/or diastolic BP (DBP) equal to or above 90 mm Hg. Hypertension is responsible for at least 45% of deaths due to heart disease, and 51% of deaths due to stroke. Globally CVD accounts for approximately 17 million deaths a year, nearly one third of the total. Out of these, complications of hypertension account for 9.4 million deaths worldwide every year.[14] Overall the prevalence of hypertension appears to be around 30–45% of the general population, with a steep increase with ageing.[15] Therefore, arterial hypertension is a serious health problem in developed and developing countries, also from an economic point of view since it causes an elevated cost for governments. It has been estimated that, at population level, a reduction in SBP of only 2 mm Hg would result in a 6% reduction in fatal stroke, and a 4% reduction in fatal coronary heart disesase.[16]

2.1. Physiological control of blood pressure

Normal levels of both SBP (120 mm Hg) and DBP (80 mm Hg) are particularly important for the efficient function of vital organs such as the heart, brain and kidneys and for overall health and wellbeing. Short- and long-term control of BP is accomplished by a number of different interacting mechanisms. Short-term control occurs primarily through the effects of the autonomic nervous system on

total peripheral vascular resistance and capacitance, and on cardiac pumping ability. Long-term control is performed by multiple nervous and hormonal controls, and by local control systems within the kidneys that regulate their excretion of salt and water.[17] Among them, the renin-angiotensin system (RAS), kallikrein-kinin system, the endothelin (ET) system and the natriuretic peptide (NP) system have been associated with long-term BP control. These systems generate a variety of vaso-regulatory peptides that collectively modulate BP, and also fluid and electrolyte balance, via membrane bound receptors located on different tissues. Hence, the main components of these systems are the targets for hypertension drug treatments and also for food-derived bioactive peptides.

The RAS pathway is one of the main targets for the treatment of hypertension, and its inhibition at three possible levels, ACE, upstream renin activity or downstream angiotensin type 1 (AT1) receptors is the pharmacological basis for commonly used antihypertensive drugs.[18] The kallikrein-kinin system represents a metabolic cascade that triggers the release of vasoactive kinins, among which the vasodilatory nonapeptide bradykinin is known best.[19] Figure 1 shows the components and functional steps by which the RAS and kallikrein-kinin system help to regulate arterial pressure.[20] As can be seen both systems are connected by ACE which degrades angiotensin I (Ang I) and bradykinin.

Another peptidic system, the ET system, has also an increasingly recognized role in BP regulation, and has also been targeted for hypertension drug treatment.[21] The synthesis of ET parallels that of the previously described peptide systems in that a precursor polypeptide, preproendothelin (preproET-1) is sequentially cleaved to generate the active form, ET-1 which has powerful

vasoconstrictor and pressor properties.[22] Figure 2 shows the regulation of blood pressure through the ET system.

The NP system consists primarily of three well-characterized peptides atrial NP (ANP), B-type NP (BNP) and C-type NP (CNP). The biological effects of these three peptides include among others natriuresis, vasodilatation and inhibition of the RAS. The NPs are cleared from the circulation via enzymatic degradation by neutral endopeptidase (NEP), a zinc-dependent membrane bound endopeptidase which is also critical for the processing and catabolism of Ang I, bradykinin and ET-1. Since many substrates for NEP are peptides with vasoactive and diuretic/natriuretic actions, NEP inhibitors have been examined as a potential therapeutic modality for hypertension treatment.[23]

2.2. Lifestyle interventions for hypertension management

The prevention of arterial hypertension development through the diet has received increasing interest, and several studies support a valid association of a limited number of dietary factors and dietary patterns with CVDs.[24] A diet low in saturated and total fat and rich in fruit, vegetables and low-fat dairy products substantially lowered BP in the Dietary Approaches to Stop Hypertension (DASH) Trial.[25] More recently, interest has grown into macronutrients intake, including dietary protein.[26] Several studies evidence a beneficial effect of protein intake on BP, specifically for plant and milk protein.[27-29] However in some epidemiological studies no inverse relation between high protein intake and BP has been seen.[30] The antihypertensive mechanism of protein is still unknown, but one possibility is the degradation of protein into peptides that have antihypertensive effects. In this context, an inverse association between low fat dairy intake and BP was found[31,

[32] and linked to bioactive peptides[33, 34] . However dairy products also contain minerals such as calcium, potassium or magnesium, and vitamins that may individually or in combination reduce BP.[35-38]

3. Milk protein-derived antihypertensive peptides

Milk proteins are the most important source of bioactive peptides, although other animal as well as plant proteins also contain potential bioactive sequences.[2] The primary and secondary structures of major human and bovine milk proteins are well characterized and the potential bioactivities of peptides released from these proteins are currently a subject of intensive research worldwide. There is now a considerable amount of scientific data to demonstrate that a wide range of milk peptides can regulate specific physiological functions in experimental animals and humans.[39-42] Undoubtedly, those with BP-lowering effects are receiving increasing attention as an alternative to drugs in the control of systemic BP and prevention of associated CVD events.[4-6, 43] These peptides might interact with the main components of BP regulatory pathways, such as RAS, kallikrein-kinin system, ET system and NP system, but nowadays ACE is the main target for antihypertensive milk-derived peptides developed as an alternative to drugs.[6]

The most extensively studied milk-derived antihypertensive peptides are the so-called lactotripeptides of sequences VPP and IPP, which can be obtained from casein by means of either milk fermentation[44] or enzymatic hydrolysis using microbial proteases.[45] In contrast to the published data from animal studies in which both lactotripeptides have shown clear antihypertensive effects in different hypertension models,[46] human data are more contradictory. Significant decreases of 4.8 mm Hg in SBP and 2.2 mm Hg in DBP were found in a meta-analysis which included 12 clinical trials,[33] in accordance with a similar meta-

analysis performed by Pripp.[47] However, in further clinical trials several authors did not find any significant effect either on SBP or DBP by treatment with lactotripeptide-containing products.[48-50] Finally, a recent meta-analysis of small doses of lactotripeptides on 19 randomized, placebo-controlled clinical intervention trials showed an overall BP lowering effect (-4.0 mm Hg for SBP; -1.0 mm Hg for DBP), although a positive effect was not reported in all the studies.[51] These contradictory data were reflected in a recent Scientific Opinion of the European Food Safety Authority on the substantiation of health claims related to IPP and VPP and maintenance of normal BP, which stated that there was no convincing evidence for a mechanism by which these widely studied bioactive peptides could exert the claimed effect.[52]

4. Lactoferrin and its derived bioactive peptides

LF is an 80 kDa iron-binding glycoprotein of the transferrin family, which was first fractionated as an unknown ‘‘red fraction’’ from cows’ milk in 1939.[53] Later on, the red protein from both human and bovine milk was defined as a transferrin-like glycoprotein.[54, 55] In mammals, LF is present in milk as a minor component of the whey fraction and also in other exocrine secretions and neutrophil granules. Nowadays it has become evident that oral administration of LF exerts several beneficial effects on the health of humans and animals, including anti-infective, anticancer, and anti-inflammatory effects. With regard to the effects of LF on BP, it has been described that chronic administration of LF strongly reduced the BP and improved antioxidant capacity in a rat model of dexamethasone-induced hypertension, suggesting that the antioxidant effect might play a role in the antihypertensive action of LF.[56] Moreover LF has an endothelial NO-dependent

hypotensive effect in rats, which is also possibly mediated by the central opioidergic system.[57] Basic research and technological aspects of the application of LF have been extensively reviewed.[10-12, 58, 59]

With respect to LF-derived peptides, the most well-known is lactoferricin B (LfcinB) which can be released from bovine LF through proteolysis by pepsin under acidic conditions,[60] a reaction that occurs naturally in the stomach.[61, 62] LfcinB is a 25-amino acid cationic antimicrobial peptide with an amphipathic, antiparallel β-sheet structure.[63] It has a single disulfide bond and no iron-binding capacity. Interestingly, LfcinB not only retains but improves the activities of LF. LfcinB possesses strong antimicrobial[64-67] and weak antiviral[68, 69] activities and it also has potent antitumoral and immunological properties[70]. Moreover, shorter derivatives of LfcinB, which are devoid of the disulfide bond, also exhibit antimicrobial activity.[71-75] Research on LfcinB and related peptides which are various short-length and amino acid-substituted peptides has been summarized in several comprehensive reviews.[70, 76, 77]

Despite the *in silico* analysis of Vermeirssen *et al*.[13] where LF stood out as a promising source of ACE-inhibitory peptides and the multifunctionality showed by LfcinB and related sequences, no attempts had been done to characterize the potential antihypertensive effects of LF-derived peptides until the antihypertensive effect after intravenous injection in spontaneously hypertensive rats (SHR) of the ACE-inhibitory sequence LRPVAA was described.[78] Later on, other LF-derived peptides with oral BP-lowering effects in SHR were reported. These studies include both short-term and long-term administration of potential antihypertensive peptides, and some of them include as well the effect of antihypertensive peptides on the SBP of the normotensive control Wistar–Kyoto

(WKY) rat strain. Although initially characterized as *in vitro* ACE inhibitors, further studies including *ex vivo* functional assays and *in vivo* approaches showed that these peptides might act on several molecular targets as explained below.

5. Mechanisms of action of lactoferrin-derived antihypertensive peptides

5.1 Effects on the renin-angiotensin system

5.1.1. ACE inhibition

In vitro ACE inhibitory effects. ACE-inhibitory potency is expressed as the IC_{50} value, or concentration needed to inhibit 50% of ACE activity. Methods based on spectrophotometric or fluorimetric detection as well as high-performance liquid chromatography (HPLC) assays employing different peptide analogs as substrates have been described.[5] Also the natural ACE substrates Ang I or bradykinin can be used to characterize ACE-inhibitory activity although differences depending on the substrate used in the *in vitro* determinations have been described.[79]

In vitro ACE-inhibitory sequences derived from different regions of the LF sequence, including its antimicrobial domain LfcinB, have been characterized (Table 1).[80-82] The ACE-inhibitory potencies of LF-derived peptides varied over a 300-fold range, with IC_{50} values of the same order of magnitude as those reported for ACE-inhibitory peptides derived from different milk proteins.[4] The two most potent LF-derived peptides corresponded to sequences LIWKL (IC_{50} = 0.47 μM)[82] and LRP (IC_{50} = 0.35 μM)[80]. Of note, their IC_{50} values are at least ten-fold lower than those described for the casein-derived antihypertensive tripeptides VPP (IC_{50} = 9.0 μM) and IPP (IC_{50} = 5.0 μM).[83] Interestingly the short-term

antihypertensive effect of both LF-derived sequences in SHRs remained significant up to 24h post-administration.[80, 82]

Despite the pioneering studies about ACE-inhibitory peptides derived from snake venom and their structural analogues, which showed the importance of the C-terminal dipeptide hydrophobic sequences of ACE substrates and inhibitors,[84-86] the effect of primary structure on potency is not fully understood. Quantitative structure-activity modeling of ACE-inhibitory peptides derived from milk proteins has shown no relationship between N-terminal structure and inhibitory potency.[87] By contrast, when we characterized LfcinB-derived peptides,[81] the sequences $LfcinB_{19-25}$, $LfcinB_{18-25}$ and $LfcinB_{17-25}$, derived from elongations at the N-terminal end of $LfcinB_{20-25}$ showed higher *in vitro* potency than the parental one, suggesting a relationship between N-terminal sequence and inhibitory activity. The four above-mentioned peptides have positively-charged R as the C-terminal residue, which has been described to contribute substantially to ACE inhibitory potency in casein-derived peptides,[88] although it does not fit with the rule proposed about residues being preferred in ultimate position (W, Y, F, P, I, A, L, M) of ACE inhibitors and substrates.[85]

The different ACE inhibitory potency found between $LfcinB_{17-25}$ and $LfcinB_{17-24}$ reinforces the importance of the R residue at the C-terminal position, whereas the differences between $LfcinB_{17-31}$ and $LfcinB_{17-32}$ support the role of C-terminal P residue in enhancing inhibition.[85] Interestingly several LF-derived sequences identified from a hydrolysate obtained either with pepsin (LNNSRAP)[82] or produced by the yeast *Kluyveromyces marxianus* (DPYKLRP, PYKLRP, YKLRP and GILRP)[80] also have a C-terminal P residue although different inhibitory potencies were found (IC_{50} values from 10.2 to 105.3 μM). The

yeast proteolytic system produced the set of sequences DPYKLRP, PYKLRP and YKLRP differing in the amino acidic residue at the N-terminal end, and together with the sequence GILRP share the C-terminal tripeptide LRP. Remarkably LRP, which can be found in three different regions of LF sequence, was pointed out as the sequence responsible of the *in silico* high ACE-inhibitory activity of different peptide sequences in LF, and in accordance with our results, an IC_{50} value of 0.27 μM was described for the tripeptide.[13] Including the peptides KLRP and LRP in the study, sequence-inhibitory potency relationships could be established and suggested that N-terminal elongations to the sequence LRP decreased *in vitro* inhibitory potency.[80] Moreover elongations at the C-terminal end of the tripeptide also provoked a decrease of inhibitory potency since an IC_{50} value of 4.14 μM was previously described for the sequence LRPVAA.[78]

However, as described for many food-derived ACE-inhibitory peptides, *in vitro* inhibitory potencies of LF-derived peptides might not result in higher antihypertensive effects as can be seen in Table 1. Despite the *in vitro* ACE-inhibitory activity showed by LfcinB-derived peptides, in the *in vivo* experiments only $LfcinB_{20\text{-}25}$, with the higher IC_{50} value of all of them, showed a moderate antihypertensive effect (-16.7 ± 3.2 mm Hg) in SHR, that was 7.7 % reduction from baseline SBP.[81] Peptides derived from other LF regions showed similar behavior on *in vivo* assays (from -13.2 mm Hg to -26.8 mm Hg) than that expected from *in vitro* potencies (from 0.35 μM to 105.3 μM). Although the IC_{50} values of LF-derived peptides were by far higher than that of ACE-inhibitory drug captopril (0.022 μM),[89] in the conditions tested, oral administration of DPYKLRP, LRP and LIWKL resulted in a significant decrease in SBP (13.4% reduction from baseline for DPYKLRP and LRP; 12.1% reduction for LIWKL) similar to that of captopril

(14% reduction). As occurred after captopril administration, the antihypertensive effect of these three LF-derived sequences remained significant for up to 24 h post-administration. Moreover, the BP-lowering effect after intravenous injection in SHR of LRPVAA was about 210% of that induced by captopril.[78] These results are also in agreement with previously reported antihypertensive effects of other food-derived peptides, which might possess higher *in vivo* effects than expected from *in vitro* inhibitory potencies probably due to their higher affinity to target tissues and their slower elimination.[90] Since ACE is a peptidase with broad substrate specificity, degradation of bioactive peptides by ACE has been argued to explain the lack of antihypertensive effect in SHR of some *in vitro* inhibitory peptides. This led to classify ACE-inhibitory peptides into three groups: inhibitor type, of which IC_{50} values are not affected by pre-incubation with ACE; the substrate type, peptides that are hydrolyzed by ACE to give peptides with a weaker activity, and the prodrug-type inhibitor, peptides that are converted to true inhibitors by ACE or other gastrointestinal proteases. Ideally, only peptides belonging to the inhibitor or prodrug-type might exert antihypertensive activities after oral administration in SHR.[91]

The lack of correlation between the *in vitro* ACE-inhibitory activity and the *in vivo* antihypertensive effect was also observed when characterizing the antihypertensive effects of complex LF hydrolysates (LFH) obtained either by enzymatic hydrolysis with different proteases such as pepsin, proteinase K and trypsin[82, 92] or by proteolytic dairy yeasts growing in LF as sole nitrogen source.[93] LFH were subjected to ultrafiltration through 3 kDa cut-off membranes and the resulting permeates (LFH < 3 kDa) inhibited ACE with IC_{50} values from 1.3 μg/mL (proteinase K LFH) to 140.2 μg/mL (*Kluyveromyces lactis* LFH) (see Table 1).

ACE inhibition by LFHs was comparable to previously reported values of other food protein-derived hydrolysates.[6, 94, 95] The antihypertensive effects of the LFHs in SHR did not correlate with their *in vitro* ACE effects. Remarkably trypsin LFH one of the most potent LFHs did not produce significant changes in SBP of SHR. By contrast yeast LFHs produced a significant BP-lowering effect after oral administration to SHR, although the effect was transient and less pronounced than the effect produced by administration of captopril. Pepsin LFH significantly reduced SBP and maintained the effect up to 24 h post-administration as observed for captopril.

Ex vivo inhibitory effects on ACE-dependent vasoconstriction. One step further in understanding the mechanisms underlying the antihypertensive effects of ACE-inhibitory peptides is to gain functional evidence for the ACE-inhibitory effects of peptides in vascular tissue. This can be assessed by *ex vivo* assays using isolated arteries since local formation of Ang II from Ang I by ACE present in the arterial wall is necessary for induction of contraction by Ang I, which lacks vasoactive effects by itself.[96, 97]

LF-derived peptides and hydrolysates showed different inhibitory effects on Ang I-induced contractions (Table 1). Peptide pre-incubation induced significant inhibitions when compared to the control with the exception of LfcinB$_{19-25}$, WQ and LNNSRAP. Regarding hydrolysates, pepsin LFH but not proteinase K LFH inhibited ACE-dependent vasoconstriction at a concentration of 100 µg/mL.[92] Non-hydrolysed LF did not provoke any inhibitory effect on Ang I-induced vasoconstriction while a non-ultrafiltered pepsin LFH only achieved such effect at a concentration of 1350 µg/mL[98] pointing out that the *ex vivo* ACE-inhibitory effect may be mainly attributable to peptide components with molecular

masses lower than 3 kDa. Representative recordings of *ex vivo* inhibitory effect on ACE-dependent vasoconstriction in rabbit isolated arteries by the well-known ACE inhibitor captopril and $LfcinB_{17-31}$ can be seen in Figure 3. Reduction of Ang I-induced vasoconstriction of thoracic aorta preparations after treatment with the casein derived ACE-inhibitory and antihypertensive peptide MKP at 100 μg/mL has been recently described.[99] Interestingly radiolabeled ^{14}C-MKP orally administered to SHR was absorbed and moved into plasma, suggesting that its ACE-inhibitory activity might contribute to induce the antihypertensive effect *in vivo*.[99]. In our *ex vivo* assays, LF-derived peptides showed inhibition of vasoconstriction at lower concentrations (20 μM in the assay corresponding to 11-42 μg/mL) than that described for MKP, suggesting the higher *ex vivo* ACE-inhibitory potency of LF-derived peptides.

As a general trend there was no correlation between *in vitro* potency and effect on ACE-dependent vasoconstriction of LF-derived peptides (see Table 1); for instance, $LfcinB_{19-25}$ with a high *in vitro* inhibitory potency (IC_{50} 2.3 ± 0.2 μM) did not show any effect on ACE-dependent vasoconstriction in contrast to $LfcinB_{20-25}$, $LfcinB_{17-31}$ and $LfcinB_{17-22}$ that had *in vitro* IC_{50} values approximately 10-fold higher.[81] Also, RPYL and LIWKL showed similar inhibitory effects on ACE-dependent vasoconstriction (14% and 22% response reduction over the control conditions, respectively) despite their 100-fold different *in vitro* potencies (IC_{50} values of 56.5 and 0.47 μM, respectively). Although this discrepancy deserves further research, a methodological detail could at least in part account for it, since *in vitro* tests were carried out with porcine ACE, while rabbit arteries were used in *ex vivo* tests, and variations in the inhibition profiles of ACE from different species have been reported.[100] Moreover, the fact that the

antihypertensive sequences WQ and LNNSRAP as well as proteinase K LFH did not show any inhibitory effect on ACE-dependent vasoconstriction suggests a mechanism for the *in vivo* antihypertensive action other than inhibition of ACE-related vasoactive effects. By contrast, the antihypertensive effect of pepsin LFH, $LfcinB_{20\text{-}25}$, RPYL and LIWKL might be due to *in vivo* ACE inhibition and subsequent reduction of Ang I-induced vascular tone.

In vivo ACE inhibition. As part of the homeostatic mechanism responsible for the maintenance of normal BP and electrolyte balance, ACE is a key component of the RAS which main function is to cleave Ang I to Ang II and degrade bradykinin (Figure 1). Therefore, *in vivo* ACE-inhibitory effect can be assessed by measuring tissue membrane-anchored or soluble circulating ACE activities, and confirmed by measuring circulating levels of Ang II. [101]

In vivo effect of pepsin LFH on ACE activity was assessed after long-term oral administration to SHR.[102] Pepsin LFH attenuated and even reversed progression of hypertension. Results showed that serum ACE activity was reduced in pepsin LFH-treated SHR. Moreover inhibition of ACE was confirmed by the reduction in Ang II level as well as in the level of aldosterone, the adrenal endocrine component downstream Ang II in the renin-angiotensin axis,[20] thus supporting ACE inhibition as an *in vivo* mechanism for the antihypertensive effect of pepsin LFH. In addition, our results also showed that ACE inhibition induced a compensatory rise of plasma renin activity which is due to the reduction of negative feedback by Ang II.[103] This renin increase further supports ACE inhibition as the antihypertensive mechanism of pepsin LFH.

Similarly, the acute antihypertensive effects of LF-derived peptides DPYKLRP and LRP might also be attributed to *in vivo* ACE inhibition because of reductions in circulating ACE activity and Ang II levels after either DPYKLRP or LRP single-intake. By contrast to that observed in pepsin LFH treatment, plasma aldosterone level of SHR was not significantly affected by any of the single-dose peptide treatments.[80] A reduction of circulating Ang II levels accompanied the BP-lowering effects in SHR after long-term oral administration of the egg-derived ACE-inhibitory peptides IQW and LKP, suggesting RAS regulation through ACE inhibition.[104] The egg-derived tripeptides decreased plasma Ang II levels by approximately 50 % after long-term treatment, but ACE activity was not measured. In our short-term *in vivo* experiments with the LF-derived sequence DPYKLRP, the maximum reductions both in ACE activity (48 %) and in Ang II level (27 %) were achieved at 1 h post administration. Moreover these effects were similar to those provoked by captopril.[80]

Another approach to assess *in vivo* ACE inhibition is the use of Ang I-induced hypertension rat models, which suitability has been shown for captopril[105] and diverse non-drug natural products.[106-108] Regarding LF-derived peptides, to investigate their *in vivo* antihypertensive mechanism, the BP-lowering effects of RPYL and DPYKLRP in Wistar rats subjected to Ang I-induced hypertension were assessed. After inducing hypertension by subcutaneous infusion of Ang I, LF-derived peptides were orally administered, and both sequences, RPYL and DPYKLRP, were able to reverse Ang I-elicited hypertension.[109] Moreover, like in SHR (see Table 1), the magnitude and duration of the antihypertensive effect were higher for DPYKLRP than for RPYL on Ang I-induced hypertension. Thus *in vivo* ACE inhibition is involved in the

antihypertensive effects of LF-derived peptides like RPYL and DPYKLRP, as suggested by two different *in vivo* experimental approaches.[80, 109]

5.1.2. Inhibition of angiotensin II receptor-mediated vasoconstriction

Inhibition of RAS at the level of AT_1 receptors is also a target for hypertension treatment (Figure 1). This effect is achieved by angiotensin receptor blocker (ARB) drugs, e.g. valsartan, because of their ability to bind to AT_1 receptors, thereby inhibiting vasoconstriction and other cellular actions of Ang II.[110, 111]

Functional *ex vivo* assays using isolated arteries are also useful to study the inhibitory effects on Ang II-induced, AT_1 receptor mediated vasoconstriction. The inhibitory effects of LF-derived peptides on Ang II-induced vasoconstriction have been assessed.[112] Peptides $LfcinB_{20-25}$, LIWKL and RPYL, pepsin LFH and the ARB valsartan produced significant inhibition of Ang II-induced vasoconstriction whereas captopril was included as negative control (Table 2). The degree of inhibition ranged from 21 % response reduction over the control conditions for the weakest $LfcinB_{20-25}$ to 44 % response reduction for the strongest RPYL, the concentration-dependent inhibitory effect (20-200 μM) of which was also shown. Thus, these results point to inhibition of Ang II-induced vasoconstriction as a potential mechanism also contributing along with ACE inhibition to the antihypertensive effect of LF-derived peptides.[112] Moreover, the AT_1 receptor-blocking mechanism for the inhibitory effect of RPYL on Ang II-induced vasoconstriction was also supported by the fact that the peptide induced significant inhibition of [^{125}I]-(Sar^1, Ile^8)-Ang II specific binding to both human and rabbit AT_1 receptors in a ligand-binding assay.[112] However, when assessing the BP-lowering effect of RPYL in Wistar rats subjected to Ang II-induced

hypertension, no inhibition of the vasopressor effect caused by Ang II was observed,[109] as would be expected from results found in *ex vivo* and binding assays.[112] Interestingly, DPYKLRP produced a modest reversion of Ang II-elicited hypertension. Thus, *in vivo* ACE inhibition is involved in the antihypertensive effects of LF-derived peptides like RPYL and DPYKLRP, while inhibition of AT_1 receptor-mediated vasoconstriction plays a less relevant role.[109]

5.2. Effects on the endothelin system

The ET system has also been targeted for hypertension drug treatment by means of ECE inhibition or ET receptor antagonism (Figure 2). Selective ECE inhibitors have been tested in preclinical rat models of hypertension,[113, 114] and ET receptor antagonists have been approved for pulmonary hypertension treatment and are under clinical development for systemic hypertension treatment.[22]

Since present strategies in the search of novel classes of antihypertensive drugs include the development of single compounds capable of simultaneously inhibiting more than one receptor or enzymatic activity involved in hypertension pathophysiology,[115] we developed a method to assay the effects of bioactive peptides on the ET system, which combines an *in vitro* test for the inhibitory effect on ECE activity and a functional *ex vivo* test for the inhibitory effect on ECE-dependent big ET-1-induced vasoconstriction and ECE-independent ET-1-induced vasoconstriction in rabbit isolated arteries.[116]

Significant inhibition of ECE activity, using the natural ECE substrate big ET-1 in order to obtain more functionally relevant results, were observed for seven LfcinB-derived peptides[116] and two sequences, GILRPY and REPYFGY, identified in a proteinase K LFH.[92] Also two antihypertensive hydrolysates, pepsin

LFH and proteinase K LFH, showed significant ECE-inhibitory effects in a concentration-dependent manner.[92] With respect to *ex vivo* functional assays of ECE inhibition in vascular tissue, we checked that big ET-1 induces ECE-dependent vasoconstriction, as supported by the inhibition of big ET-1-induced, but not ET-1-induced contractions with the ECE inhibitor phosphoramidon.[116] Regarding LfcinB-derived peptides, six of them inhibited big ET-1-induced, ECE-dependent vasoconstriction with good correlation with the *in vitro* inhibitory effects,[116] while two LF-hydrolysates induced inhibition when compared to control vasoconstriction. However, in contrast to *in vitro* assays of ECE inhibitory effect, pepsin LFH induced higher inhibition of vasoconstriction than that produced by the proteinase K LFH.[92] Table 3 summarizes the inhibitory effects of LF-derived peptides and hydrolysates on ECE activity and ECE-dependent vasoconstriction. Of note, *ex vivo* functional assays for ECE-independent ET-1-induced vasoconstriction showed that LF-derived peptides do not act on downstream ET_A receptors or intracellular signal transduction mechanisms leading to vasoconstriction.[112, 116]

Taken into account the effects of LfcinB-derived peptides and LFHs on ACE and ECE inhibition (see Tables 1 and 3), five of the LfcinB-derived peptides were dual vasopeptidase (ACE/ECE) inhibitors with anti-vasoconstrictor effects. Results suggest dual ACE and ECE inhibition as mechanisms involved in the antihypertensive effect observed in SHR after pepsin LFH administration, and that ECE inhibition seems to be the mechanism involved in the moderate antihypertensive effect of proteinase K LFH. *In vivo* effects of LF-derived peptides on the ET system require further research.

5.3 Effects on other blood pressure regulating pathways

Nowadays high throughput techniques offer a powerful approach for understanding the molecular basis of bioactive compounds. Regarding antihypertensive peptides, for which several molecular targets have been highlighted, global approaches represent a feasible strategy for revealing the action of these peptides through distinct BP regulating pathways. DNA microarray technology was applied for the analysis of changes in gene expression in aorta of SHR after repeated administration of casein-derived VPP and IPP.[117] Changes in gene expression were mild and the most marked differences were found in the expression of genes associated with vascular function, such as the endothelial NO synthase (eNOS) gene, the connexin 40 (gap junction 40) gene and the NF-κB (nuclear factor kappa B subunit) gene. For the arachidonic acid system, the cyclooxigenase (COX-1) gene showed a slight increase in expression after VPP and IPP administration, and related to cytokine production, the PPARγ (peroxisome proliferator activator receptor, gamma) gene showed significantly lower expression after the VPP and IPP treatment. There were no significant changes in the expression resulting from the intake of VPP and IPP, for genes associated with the RAS or with the blood coagulation system. Recently, the same technology was used to determine the molecular mechanism of the antihypertensive effect of milk fermented by *Lactobacillus helveticus*.[118] It was found that hypertension-associated genes differentially expressed in the left ventricle of SHR were related to NO synthesis, cell proliferation, ET binding and blood clot breakdown. Specifically, regulation of eNOS, PPARγ and ET_A receptor genes could be responsible for the antihypertensive response provoked by fermented milk treatment.

Regarding LF-derived antihypertensive peptides, the effects of pepsin LFH on the expression of a panel of genes related to hypertension were evaluated by RT-PCR in human umbilical vein endothelial cells (HUVEC).[119] Pepsin LFH treatment significantly affected the expression of genes encoding proteins involved in the NO pathway such as NO synthase trafficking (NOSTRIN) and soluble guanylate cyclase 1 α3 subunit (GUCY1A3). NOSTRIN is a protein which modulates subcellular distribution of eNOS and thus NO release. Its overexpression promotes the translocation of eNOS from the plasma membrane to intracellular vesicles, with a concomitant reduction in eNOS enzyme activity and inhibition of NO synthesis.[120] Conversely, decreased NOSTRIN expression also influences eNOS subcellular localization and contributes to increase NO levels in endothelial cells.[121] We found that NOSTRIN expression was significantly downregulated after LFH treatment for 24 h and that this result was consistent with a reduced protein expression detected by immunoblot analysis and an increased NO production. In addition, our results also showed upregulated expression of soluble guanylate cyclase GC (GUCY1), the physiological target of NO[122] and presumably the most relevant molecular target for NO-releasing drugs in human cardiovascular therapy.[123] Furthermore, expression of the PTGS2/COX-2 gene encoding prostaglandin-endoperoxide synthase 2, a key component of prostaglandin synthesis, was significantly increased following pepsin LFH treatment in HUVEC.[119] In healthy humans, COX-2 generates mainly prostacyclin, a potent vasodilator and platelet inhibitor.[124] Moreover, it has been described that ACE inhibitors increase expression of COX-2 and prostacyclin levels in different experimental models [125] thus suggesting that COX-2 induction, like that provoked by LFH, may potentiate vasodilator activity.

Despite inhibition of ACE activity by pepsin LFH was shown in cultured HUVEC supernatants, no effect on ACE mRNA levels was observed,[119] in agreement to that found in aorta of SHR after administration of VPP and IPP.[117] Despite ECE has been shown as a molecular target for pepsin LFH,[92] we did not find significant changes in ECE relative mRNA level or in expression of other genes associated with the ET system.[119] Time-course effect of pepsin LFH in HUVEC on mRNA levels of genes associated with RAS and ET system are necessary to discard any regulation at the mRNA level at shorter exposure times.

Although studies assessing the antihypertensive mechanisms of action of food protein-derived peptides by means of global approaches are still scarce, they point to different molecular targets in the NO and prostaglandin pathways. Moreover these studies reveal the complexity of effects exerted by antihypertensive LF-derived peptides opening avenues for the better understanding of their BP-lowering effects. Undoubtedly the application of these techniques to different *in vitro* and in *vivo* models will considerably extend the current evidence regarding LF-derived antihypertensive peptides and hypertension management.

6. Conclusions and perspectives

Data reported here demonstrate the potential application of LF-derived peptides released from different regions of the protein in the control of hypertension. These bioactive peptides can be obtained by enzymatic hydrolysis, but also using GRAS (Generally Recognized As Safe) proteolytic dairy yeasts. Different experimental approaches, including *in vitro*, *ex vivo* and *in vivo* assays point out the RAS pathway, mainly ACE but also AT_1 receptors, as a target for LF-derived

antihypertensive peptides. Renin inhibition, recently highlighted as a potential mechanism for other food-derived antihypertensive peptides,[9] has not been directly assessed for LF-derived sequences but remarkably an increase of plasma renin activity in SHR after long-term treatment with pepsin LFH as a compensatory effect of ACE inhibition was observed. Further research is needed to establish the role of renin inhibition in the antihypertensive effect of LF-derived peptides. Results suggest that ECE inhibition but not ET_A receptor blocking mechanism might also contribute to the *in vivo* antihypertensive effect of LF-derived peptides some of which might act as dual ACE/ECE vasopeptidase inhibitors. Hence, involvement of the ET system in the BP-lowering effects merits future studies. Finally, the HUVEC *in vitro* model showed that pepsin LFH was able to increase NO production and modify the expression of hypertension related genes, suggesting NOSTRIN as a target for LF-derived peptides. The potential contribution of prostaglandins was also suggested. Figure 4 summarizes the mechanisms by which LF-derived peptides might exert antihypertensive effects.

All together these studies identify several molecular targets for LF-derived peptides other than ACE inhibition and highlight the multiple mechanisms underlying BP-lowering effects, reinforcing the great value of LF as an effective source of multifunctional antihypertensive peptides. Nevertheless clinical trials are mandatory to demonstrate the antihypertensive effects of LF-derived peptides already observed in SHR and to obtain further evidence of the participation of such peptides in different BP regulatory pathways. Finally, a last but not least important issue is related to the bioavailability properties of LF-derived antihypertensive peptides. Certainly identification of the peptide active

fragment able to reach the molecular target in the organism as well as the evaluation of absorption, distribution, metabolism and excretion are essential.

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References

1. E. G. de Mejia and V. P. Dia, *Cancer Metastasis Rev.*, 2010, **29**, 511-528.
2. R. Hartmann and H. Meisel, *Curr Opin Biotech*, 2007, **18**, 163-169.
3. H. Korhonen and A. Pihlanto, *Int Dairy J*, 2006, **16**, 945-960.
4. F. De Leo, S. Panarese, R. Gallerani and L. R. Ceci, *Curr Pharm Des*, 2009, **15**, 3622-3643.
5. B. Hernández-Ledesma, M. del Mar Contreras and I. Recio, *Adv Colloid Interface Sci*, 2011, **165**, 23-35.
6. D. Martinez-Maqueda, B. Miralles, I. Recio and B. Hernandez-Ledesma, *Food Func*, 2012, **3**, 350-361.
7. K. Majumder and J. Wu, *Int J Mol Sci*, 2014, **16**, 256-283.
8. C. Marques, M. M. Amorim, J. O. Pereira, M. Estevez Pintado, D. Moura, C. Calhau and H. Pinheiro, *Curr Pharm Des*, 2012, **18**, 4706-4713.
9. C. C. Udenigwe and A. Mohan, *J Func Foods*, 2014, **8**, 45-52.
10. S. Farnaud and R. W. Evans, *Mol Immunol*, 2003, **40**, 395-405.
11. M. Tomita, H. Wakabayashi, K. Shin, K. Yamauchi, T. Yaeshima and K. Iwatsuki, *Biochimie*, 2009, **91**, 52-57.
12. H. Wakabayashi, K. Yamauchi and M. Takase, *Int Dairy J*, 2006, **16**, 1241-1251.
13. V. Vermeirssen, A. van der Bent, J. Van Camp, A. van Amerongen and W. Verstraete, *Biochimie*, 2004, **86**, 231-239.
14. WHO, *WHO/DCO/WHD/2013.2*, 2013.
15. G. Mancia, R. Fagard, K. Narkiewicz, J. Redón, A. Zanchetti, M. Böhm, T. Christiaens, R. Cifkova, G. De Backer, A. Dominiczak, M. Galderisi, D. E. Grobbee, T. Jaarsma, P. Kirchhof, S. E. Kjeldsen, S. Laurent, A. J. Manolis, P. M. Nilsson, L. M. Ruilope, R. E. Schmieder, P. A. Sirnes, P. Sleight, M. Viigimaa, B. Waeber and F. Zannad, *J Hypertens*, 2013, **31**, 1281-1357.
16. P. K. Whelton, J. He, L. J. Appel, J. A. Cutler, S. Havas, T. A. Kotchen, E. J. Roccella, R. Stout, C. Vallbona, M. C. Winston and J. Karimbakas, *JAMA*, 2002, **288**, 1882-1888.
17. A. W. Cowley, *Physiol Rev*, 1992, **72**, 231-300.
18. G. Fragasso, F. Maranta, C. Montanaro, A. Salerno, C. Torlasco and A. Margonato, *Expert Opin Ther Tar*, 2012, **16**, 179-193.
19. M. E. Moreau, N. Garbacki, G. Molinaro, N. J. Brown, F. Marceau, ccedil, ois and A. Adam, *J Pharmacol Sci*, 2005, **99**, 6-38.
20. R. M. Carey and H. M. Siragy, *Endocr Rev*, 2003, **24**, 261-271.
21. E. L. Schiffrin, *Vas Pharmacol*, 2005, **43**, 19-29.
22. V. S. Prasad, C. Palaniswamy and W. H. Frishman, *Cardiol Rev*, 2009, **17**.
23. S. Mangiafico, L. C. Costello-Boerrigter, I. A. Andersen, A. Cataliotti and J. C. Burnett, *Eur Heart J*, 2012.
24. A. Mente, L. de Koning, H. S. Shannon and S. S. Anand, *Arch Intern Med*, 2009, **169**, 659-669.
25. L. J. Appel, T. J. Moore, E. Obarzanek, W. M. Vollmer, L. P. Svetkey, F. M. Sacks, G. A. Bray, T. M. Vogt, J. A. Cutler, M. M. Windhauser, P.-H. Lin, N. Karanja, D. Simons-Morton, M. McCullough, J. Swain, P. Steele, M. A. Evans, E. R. Miller and D. W. Harsha, *New Engl J Med*, 1997, **336**, 1117-1124.

26. L. J. Appel, F. M. Sacks, V. J. Carey, E. Obarzanek, J. F. Swain, E. R. Miller, P. R. Conlin, T. P. Erlinger, B. A. Rosner, N. M. laranjo, J. Charleston, P. McCarron and L. M. Bishop, *JAMA*, 2005, **294**, 2455-2464.
27. W. Altorf – van der Kuil, M. F. Engberink, E. J. Brink, M. A. van Baak, S. J. L. Bakker, G. Navis, P. van 't Veer and J. M. Geleijnse, *PLoS ONE*, 2010, **5**, e12102.
28. W. Altorf - van der Kuil, M. F. Engberink, M. M. Vedder, J. M. A. Boer, W. M. M. Verschuren and J. M. Geleijnse, *PLoS ONE*, 2012, **7**, e30582.
29. J. He, M. R. Wofford, K. Reynolds, J. Chen, C.-S. Chen, L. Myers, D. L. Minor, P. J. Elmer, D. W. Jones and P. K. Whelton, *Circulation*, 2011, **124**, 589-595.
30. I. M. Hajjar, C. E. Grim, V. George and T. A. Kotchen, *Arch Intern Med*, 2001, **161**, 589-593.
31. M. F. Engberink, M. A. Hendriksen, E. G. Schouten, F. J. van Rooij, A. Hofman, J. C. Witteman and J. M. Geleijnse, *Am J Clin Nutr*, 2009, **89**, 1877-1883.
32. R. A. Ralston, J. H. Lee, H. Truby, C. E. Palermo and K. Z. Walker, *J Human Hypertens*, 2012, **26**, 3-13.
33. J. Y. Xu, L. Q. Qin, P. Y. Wang, W. Li and C. Chang, *Nutrition*, 2008, **24**, 933-940.
34. T. Jauhiainen and R. Korpela, *J Nutr*, 2007, **137**, 825S-829S.
35. T. B. Drueke, *Am J Hypertens*, 1999, **12**, 96-98.
36. J. M. Geleijnse, F. J. Kok and D. E. Grobbee, *J Human Hypertens*, 2003, **17**, 471-480.
37. S. Mizushima, F. P. Cappuccio, R. Nichols and P. Elliott, *J Human Hypertens*, 1998, **12**, 447-453.
38. S. S. Soedamah-Muthu, L. D. M. Verberne, E. L. Ding, M. F. Engberink and J. M. Geleijnse, *Hypertension*, 2012, **60**, 1131-1137.
39. C. Beermann and J. Hartung, *Food Func*, 2013, **4**, 185-199.
40. B. Hernandez-Ledesma, M. J. Garcia-Nebot, S. Fernandez-Tome, L. Amigo and I. Recio, *Int Dairy J*, 2014, **38**, 82-100.
41. C. Muro Urista, R. Álvarez Fernández, F. Riera Rodriguez, A. Arana Cuenca and A. Téllez Jurado, *Food Sci Technol Int*, 2011, **17**, 293-317.
42. R. Nagpal, P. Behare, R. Rana, A. Kumar, M. Kumar, S. Arora, F. Morotta, S. Jain and H. Yadav, *Food Func*, 2011, **2**, 18-27.
43. B. A. Murray and R. J. FitzGerald, *Curr Pharm Des*, 2007, **13**, 773-791.
44. Y. Nakamura, N. Yamamoto, K. Sakai and T. Takano, *J Dairy Sci*, 1995, **78**, 1253-1257.
45. S. Mizuno, S. Nishimura, K. Matsuura, T. Gotou and N. Yamamoto, *J Dairy Sci*, 2004, **87**, 3183-3188.
46. P. Jäkälä and H. Vapaatalo, *Pharmaceuticals*, 2010, **3**, 251-272.
47. A. H. Pripp, *Food Nutr Res*, 2008, **52**.
48. M. F. Engberink, E. G. Schouten, F. J. Kok, L. A. J. van Mierlo, I. A. Brouwer and J. M. Geleijnse, *Hypertension*, 2008, **51**, 399-405.
49. K. van der Zander, M. L. Bots, A. A. A. Bak, M. M. G. Koning and P. W. de Leeuw, *Am J Clin Nutr*, 2008, **88**, 1697-1702.
50. L. A. J. van Mierlo, M. M. G. Koning, K. van der Zander and R. Draijer, *Am J Clin Nutr*, 2009, **89**, 617-623.
51. A. M. Turpeinen, S. Järvenpää, H. Kautiainen, R. Korpela and H. Vapaatalo, *Ann Med*, 2013, **45**, 51-56.

52. EFSA Panel on Dietetic Products Nutrition and Allergies*, EFSA J*, 2012, **10**, 2715-2737.
53. M. Sørensen and S. P. L. Sørensen, *Comp Rendus Trav Lab Carlsberg*, 1939, **23**, 55-99.
54. M. L. Groves, *J Am Chem Soc*, 1960, **82**, 3345–3350.
55. B. Johansson, *Acta Chem Scand*, 1960, **14**, 510-512.
56. L. Safaeian and H. Zabolian, *ISRN Pharmacol*, 2014, **2014**, 943523.
57. K.-i. Hayashida, T. Takeuchi, T. Ozaki, H. Shimizu, K. Ando, A. Miyamoto and E. Harada, *Am J Physiol Regul Integr Comp Physiol*, 2004, **286**, R359-R365.
58. M. C. Ammons and V. Copie, *Biofouling*, 2013, **29**, 443-455.
59. H. Tsuda, K. Sekine, K.-i. Fujita and M. Iigo, *Biochem Cell Biol*, 2002, **80**, 131-136.
60. M. Tomita, W. Bellamy, M. Takase, K. Yamauchi, H. Wakabayashi and K. Kawase, *J Dairy Sci*, 1991, **74**, 4137-4142.
61. H. Kuwata, T. T. Yip, M. Tomita and T. W. Hutchens, *BBA-Protein Struct Mol Enzym*, 1998, **1429**, 129-141.
62. H. Kuwata, T. T. Yip, K. Yamauchi, S. Teraguchi, H. Hayasawa, M. Tomita and T. W. Hutchens, *Biochem J*, 1998, **334**, 321-323.
63. P. M. Hwang, N. Zhou, X. Shan, C. H. Arrowsmith and H. J. Vogel, *Biochemistry*, 1998, **37**, 4288-4298.
64. W. Bellamy, M. Takase, H. Wakabayashi, K. Kawase and M. Tomita, *J Appl Bacteriol*, 1992, **73**, 472-479.
65. W. Bellamy, K. Yamauchi, H. Wakabayashi, M. Takase, N. Takakura, S. Shimamura and M. Tomita, *Lett Appl Microbiol*, 1994, **18**, 230-233.
66. H. Wakabayashi, S. Abe, T. Okutomi, S. Tansho, K. Kawase and H. Yamaguchi, *Microbiol Immunol*, 1996, **40**, 821-825.
67. K. Yamauchi, M. Tomita, T. J. Giehl and R. T. Ellison, *Infect Immun*, 1993, **61**, 719-728.
68. J. H. Andersen, H. Jenssen and T. J. Gutteberg, *Antivir Res*, 2003, **58**, 209-215.
69. J. H. Andersen, S. A. Osbakk, L. H. Vorland, T. Traavik and T. J. Gutteberg, *Antivir Res*, 2001, **51**, 141-149.
70. J. L. Gifford, H. N. Hunter and H. J. Vogel, *Cell Mol Life Sci*, 2005, **62**, 2588-2598.
71. J. H. Kang, M. K. Lee, K. L. Kim and K. S. Hahm, *Int J Pept Prot Res*, 1996, **48**, 357-363.
72. H. Wakabayashi, H. Matsumoto, K. Hashimoto, S. Teraguchi, M. Takase and H. Hayasawa, *Antimicrob Agents Ch*, 1999, **43**, 1267-1269.
73. M. Enrique, P. Manzanares, M. Yuste, M. Martinez, S. Valles and J. F. Marcos, *Food Microbiol*, 2009, **26**, 340-346.
74. M. Enrique, J. F. Marcos, M. Yuste, M. Martinez, S. Valles and P. Manzanares, *Int J Food Microbiol*, 2007, **118**, 318-325.
75. M. Enrique, J. F. Marcos, M. Yuste, M. Martinez, S. Valles and P. Manzanares, *Int J Food Microbiol*, 2008, **127**, 229-234.
76. M. B. Strøm, B. E. Haug, Ø. Rekdal, M. L. Skar, W. Stensen and J. S. Svendsen, *Biochem Cell Biol*, 2002, **80**, 65-74.
77. H. J. Vogel, D. J. Schibli, W. Jing, E. M. Lohmeier-Vogel, R. F. Epand and R. M. Epand, *Biochem Cell Biol*, 2002, **80**, 49-63.

78. N. Y. Lee, J. T. Cheng, T. Enomoto and I. Nakamura, *Chinese J Physiol*, 2006, **49**, 67-73.
79. J. M. Centeno, M. C. Burguete, M. Castello-Ruiz, M. Enrique, S. Valles, J. B. Salom, G. Torregrosa, J. F. Marcos, E. Alborch and P. Manzanares, *J Agric Food Chem*, 2006, **54**, 5323-5329.
80. A. Garcia-Tejedor, L. Sanchez-Rivera, M. Castello-Ruiz, I. Recio, J. B. Salom and P. Manzanares, *J Agric Food Chem*, 2014, **62**, 1609-1616.
81. P. Ruiz-Giménez, A. Ibañez, J. B. Salom, J. F. Marcos, J. J. López-Díez, S. Vallés, G. Torregrosa, E. Alborch and P. Manzanares, *J Agric Food Chem*, 2010, **58**, 6721-6727.
82. P. Ruiz-Giménez, J. B. Salom, J. F. Marcos, S. Vallés, D. Martínez-Maqueda, I. Recio, G. Torregrosa, E. Alborch and P. Manzanares, *Food Chem*, 2012, **131**, 266-273.
83. Y. Nakamura, N. Yamamoto, K. Sakai, A. Okubo, S. Yamazaki and T. Takano, *J Dairy Sci*, 1995, **78**, 777-783.
84. H. S. Cheung and D. W. Cushman, *Biochim Biophys Acta* 1973, **293**, 451-463.
85. H. S. Cheung, F. L. Wang, M. A. Ondetti, E. F. Sabo and D. W. Cushman, *J Biol Chem*, 1980, **255**, 401-407.
86. D. W. Cushman, J. Pluščec, N. J. Williams, E. R. Weaver, E. F. Sabo, O. Kocy, H. S. Cheung and M. A. Ondetti, *Experientia*, 1973, **29**, 1032-1035.
87. A. H. Pripp, T. Isaksson, L. Stepaniak and T. Sorhaug, *Eur Food Res Technol*, 2004, **219**, 579-583.
88. R. J. FitzGerald and H. Meisel, *Brit J Nutr*, 2000, **84**, 33-37.
89. M. A. Ondetti and D. W. Cushman, *Science*, 1977, **196**, 441-444.
90. H. Fujita and M. Yoshikawa, *Immunopharmacology*, 1999, **44**, 123-127.
91. H. Fujita, K. Yokoyama and M. Yoshikawa, *J Food Sci*, 2000, **65**, 564-569.
92. R. Fernández-Musoles, J. B. Salom, D. Martínez-Maqueda, J. J. López-Díez, I. Recio and P. Manzanares, *Food Chem*, 2013, **139**, 994-1000.
93. A. Garcia-Tejedor, B. Padilla, J. B. Salom, C. Belloch and P. Manzanares, *Food Res Int*, 2013, **53**, 203-208.
94. M. M. Contreras, R. Carrón, M. J. Montero, M. Ramos and I. Recio, *Int Dairy J*, 2009, **19**, 566-573.
95. M. Miguel, I. Recio, J. A. Gomez-Ruiz, M. Ramos and R. Lopez-Fandino, *J Food Protect*, 2004, **67**, 1914-1920.
96. C. Eglème, F. Cressier and J. M. Wood, *Brit J Pharmacol*, 1990, **100**, 237-240.
97. E. T. Whalley, *Naunyn-Schmiedeberg's Arch Pharmacol*, 1987, **335**, 551-554.
98. P. Ruiz-Gimenez, M. C. Burguete, M. Castello-Ruiz, J. F. Marcos, J. B. Salom, S. Valles, G. Torregrosa, E. Alborch and P. Manzanares, *Int Dairy J*, 2007, **17**, 1212-1215.
99. A. Yamada, T. Sakurai, D. Ochi, E. Mitsuyama, K. Yamauchi and F. Abe, *Food Chem*, 2015, **172**, 441-446.
100. G. Vazeux, J. Cotton, P. Cuniasse and V. Dive, *Biochem Pharmacol*, 2001, **61**, 835-841.
101. D. Coates, *Int J Biochem Cell B*, 2003, **35**, 769-773.
102. R. Fernandez-Musoles, P. Manzanares, M. C. Burguete, E. Alborch and J. B. Salom, *Food Res Int*, 2013, **54**, 627-632.

103. H. R. Brunner, J. Nussberger and B. Waeber, *J Hypertens*, 1993, **11**, S53-S58.
104. K. Majumder, S. Chakrabarti, J. S. Morton, S. Panahi, S. Kaufman, S. T. Davidge and J. Wu, *J Func Foods*, 2015, **13**, 50-60.
105. N. R. Levens, M. J. Peach, E. Darracot Vaughan JR, W. C. Weed and R. M. Carey, *Endocrinology*, 1981, **108**, 536-544.
106. J. C. Liu, J. C. Tsai, P. Chan, J. Y. H. Liu, G. N. Thomas, B. Tomlinson, M. Y. Lo and J. Y. Lin, *Life Sci*, 2003, **73**, 1543-1555.
107. K. Prasad, *Int J Angiol* 2013, **22**, 235-238.
108. H. Waghulde, M. Mohan, S. Kasture and R. Balaraman, *Int J PharmTech Res*, 2010, **2**, 60-67.
109. A. García-Tejedor, M. Castelló-Ruiz, J. V. Gimeno-Alcañíz, P. Manzanares and J. B. Salom, *J Func Foods*, 2015, **15**, 294-300.
110. D. A. Sica, *J Clin Hypertens*, 2006, **8**, 381-385.
111. A. A. Taylor, H. Siragy and S. Nesbitt, *J Clin Hypertens*, 2011, **13**, 677-686.
112. R. Fernandez-Musoles, M. Castello-Ruiz, C. Arce, P. Manzanares, M. D. Ivorra and J. B. Salom, *J Agric Food Chem*, 2014, **62**, 173-181.
113. B. Battistini, B. Ayach, S. Molez, A. Blouin and A. Y. Jeng, *Clin Sci*, 2002, **103**, 363S-366S.
114. A. J. Trapani, M. E. Beil, C. W. Bruseo, S. De Lombaert and A. Y. Jeng, *J Cardiovasc Pharm*, 2000, **36**, S40-S43.
115. B. Battistini, P. Daull and A. Y. Jeng, *Cardiovasc Drug Rev*, 2005, **23**, 317-330.
116. R. Fernández-Musoles, J. J. López-Díez, G. Torregrosa, S. Vallés, E. Alborch, P. Manzanares and J. B. Salom, *Peptides*, 2010, **31**, 1926-1933.
117. N. Yamaguchi, K. Kawaguchi and N. Yamamoto, *Eur J Pharmacol*, 2009, **620**, 71-77.
118. Y. Chen, Z. Zhong, J. Xue, J. Yang, Q. Zhang, J. Yue and H. Zhang, *Dairy Sci. Technol.*, 2015, **95**, 321-330.
119. A. García-Tejedor, J. V. Gimeno-Alcañíz, S. Tavárez, E. Alonso, J. B. Salom and P. Manzanares, *J Func Foods*, 2015, **12**, 45-54.
120. K. Zimmermann, N. Opitz, J. Dedio, C. Renné, W. Müller-Esterl and S. Oess, *P Natl Acad Sci*, 2002, **99**, 17167-17172.
121. M. E. McCormick, R. Goel, D. Fulton, S. Oess, D. Newman and E. Tzima, *Arterioscl Thromb Vasc Biol*, 2011, **31**, 643-649.
122. W. P. Arnold, C. K. Mittal, S. Katsuki and F. Murad, *P Natl Acad Sci*, 1977, **74**, 3203-3207.
123. U. Zabel, M. Weeger, M. La and H. H. H. W. Schmidt, *Biochem J*, **335**, 51-57.
124. B. F. McAdam, F. Catella-Lawson, I. A. Mardini, S. Kapoor, J. A. Lawson and G. A. FitzGerald, *P Natl Acad Sci*, 1999, **96**, 272-277.
125. K. Kohlstedt, R. Busse and I. Fleming, *Hypertension*, 2005, **45**, 126-132.

Figure captions

Fig. 1 Renin-angiotensin and kallikrein-kinin systems, from peptide precursors to receptors involved in blood pressure regulation. Sites of action of inhibitory antihypertensive drugs are depicted by the symbol ⊥. ACE, angiotensin I-converting enzyme; AT_1, angiotensin type 1 receptor; B_2, kinin type 2 receptor; NO, nitric oxide.

Fig. 2 Endothelin system, from peptide precursors to receptors involved in blood pressure regulation. Sites of action of inhibitory antihypertensive drugs are depicted by the symbol ⊥. ET, endothelin; ECE, endothelin converting enzyme; ET_A, endothelin type A receptor; ET_B, endothelin type B receptor.

Fig. 3 *Ex vivo* inhibitory effect of the lactoferrin-derived peptide $LfcinB_{17-31}$ on ACE-dependent vasoconstriction. Rabbit carotid artery segments are subjected to isometric tension recording in an organ bath, and representative recordings are shown. Top: Angiotensin I (Ang I; 1 *μ*M) induces phasic, transient contraction. Preincubation with the ACE inhibitor drug captopril (1 μM) completely abolishes Ang I-induced contraction. This inhibition is reversible after captopril wash-out, thus supporting ACE-dependence of Ang I-induced contraction. Bottom: $LfcinB_{17-31}$ (20 μM), inhibits Ang I-induced, ACE-dependent vasoconstriction. Adapted from Centeno et al., 2006.[79]

Fig. 4 Antihypertensive mechanisms of lactoferrin-derived peptides: the effects on the renin-angiotensin system, the endothelin system and pathway for nitric oxide (NO) production promote reduction of vascular tone and subsequent blood pressure downregulation. Inhibition sites are depicted by the symbol ⊥, whereas stimulation sites are depicted by the symbol ↓. ACE, angiotensin I-converting

enzyme; AT_1, angiotensin type 1 receptor; ECE, endothelin converting enzyme; ET, endothelin; ET_A, endothelin type A receptor; NOSTRIN, NO synthase trafficking.

Table 1 ACE-inhibitory potency, inhibition of ACE-dependent vasoconstriction and oral antihypertensive effects of lactoferrin (LF)-derived peptides and hydrolysates

Peptide	Sequence	IC_{50}[a]	Inhibition of vasoconstriction (%)[b]	SBP (mm Hg)[c]	Ref.
LfcinB-derived					
$LfcinB_{17-32}$	FKCRRWQWRMKKLGAP	11.0 ± 1.5	20	n.s.	81
$LfcinB_{17-31}$	FKCRRWQWRMKKLGA	25.5 ± 2.3	21	n.s.	79,81
$LfcinB_{20-25}$	RRWQWR	32.0 ± 4.9	30	-16.7 ± 3.2	81
$LfcinB_{19-25}$	CRRWQWR	2.3 ± 0.1	n.s.	n.s.	81
$LfcinB_{18-25}$	KCRRWQWR	5.8 ± 0.2	25	n.s.	81
$LfcinB_{17-25}$	FKCRRWQWR	2.9 ± 0.6	26	n.s.	81
$LfcinB_{17-24}$	FKCRRWQW	10.5 ± 0.6	18	n.s.	81
$LfcinB_{17-22}$	FKCRRW	26.7 ± 1.9	28	n.s.	81
$LfcinB_{21-23}$	RWQ	n.d.	n.d.	n.s.	81
$LfcinB_{22-23}$	WQ	n.d.	n.s.	-11.4 ± 2.7	81
LF-derived					
f(266-270)	LIWKL	0.47 ± 0.01	22	-25.3 ± 3.5	82
f(133-136)	RPYL	56.5 ± 1.9	14	-18.9 ± 2.3	82
f(232-238)	LNNSRAP	105.3 ± 6.4	n.s.	-15.3 ± 3.7	82
f(70-76)	DPYKLRP	30.5 ± 1.4	n.d.	-26.8 ± 2.4	80

f(71-76)	PYKLRP	10.2 ± 1.2	n.d.	-22.3 ± 0.9	80
f(72-76)	YKLRP	16.5 ± 0.7	n.d.	-21.1 ± 1.6	80
f(73-76)	KLRP	91.6 ± 4.0	n.d.	-13.2 ± 0.9	80
f(74-76), f(132-134), f(427-429)	LRP	0.35 ± 0.03	n.d.	-26.8 ± 1.3	80
f(130-134)	GILRP	90.7 ± 5.0	n.d.	-20.5 ± 2.9	80
LF hydrolysates					
Pepsin LFH		14.3 ± 3.3	29	-15.9 ± 3.6	82
Proteinase K LFH		1.3 ± 0.1	n.s.	-19 ± 7	92
Trypsin LFH		6.9 ± 0.2	n.d.	n.s.	92
Debaryomyces hansenii LFH		89.6 ± 3.7	n.d.	-18 ± 2	93
Kluyveromyces lactis LFH		140.2 ± 9.2	n.d.	-12 ± 1	93
Kluyveromyces marxianus LFH		50.2 ± 2.7	n.d.	-24 ± 1	93

[a]IC_{50} values are given in μM (peptides) or μg/mL (LF hydrolysates) units.
[b]Inhibition of ACE-dependent vasoconstriction expressed as the percentage with respect to control. Peptides were assayed at 20 μM and hydrolysates at 100 μg/mL.
[c]Maximum decrease in SBP after oral doses of 10 mg/kg (peptides) or 200 mg/kg (LF hydrolysates).
n.s. not significant; n.d. not determined.

Table 2 Inhibitory effects of lactoferrin-derived peptides, captopril and valsartan on angiotensin II-induced vasoconstriction

Compound	Inhibition of vasoconstriction (%)[a]
$LfcinB_{20-25}$ (20 μM)	21
LIWKL (20 μM)	30
RPYL (20 μM)	44
RPYL (200 μM)	80
Pepsin LFH (100 μg/mL)	25
Captopril (0.1 μM)	n.s.
Valsartan (10 nM)	69
Valsartan (0.1 μM)	88

[a]Inhibition of ang II-dependent vasoconstriction expressed as the percentage with respect to control.

n.s. not significant

Table 3 Inhibitory effects of lactoferrin (LF)-derived peptides and hydrolysates on endothelin-converting enzyme (ECE) activity and ECE-dependent vasoconstriction

Peptide[a]	ECE inhibition (%)[b]	Inhibition of vasoconstriction (%)[c]	Ref.
LfcinB-derived			
$LfcinB_{17-32}$	81	54.0	116
$LfcinB_{17-31}$	82	54.0	116
$LfcinB_{19-25}$	67	66.4	116
$LfcinB_{18-25}$	42	43.8	116
$LfcinB_{17-25}$	86	62.0	116
$LfcinB_{17-24}$	38	n.s.	116
$LfcinB_{17-22}$	38	54.7	116
LF-derived			
GILRPY	13	n.d.	92
REPYFGY	23	n.d.	92
LF hydrolysates			
Pepsin LFH	92	30.8	92
Proteinase K LFH	31	21.2	92

[a]Peptides were assayed at 30 μM and hydrolysates at 100 μg/mL.

[b]Percent of ECE activity inhibition with respect to a control without peptide or hydrolysate.

[c]Inhibition of ECE-dependent vasoconstriction expressed as the percentage with respect to that of control.

n.s. not significant; n.d. not determined.

Figure 1 Manzanares et al 2015

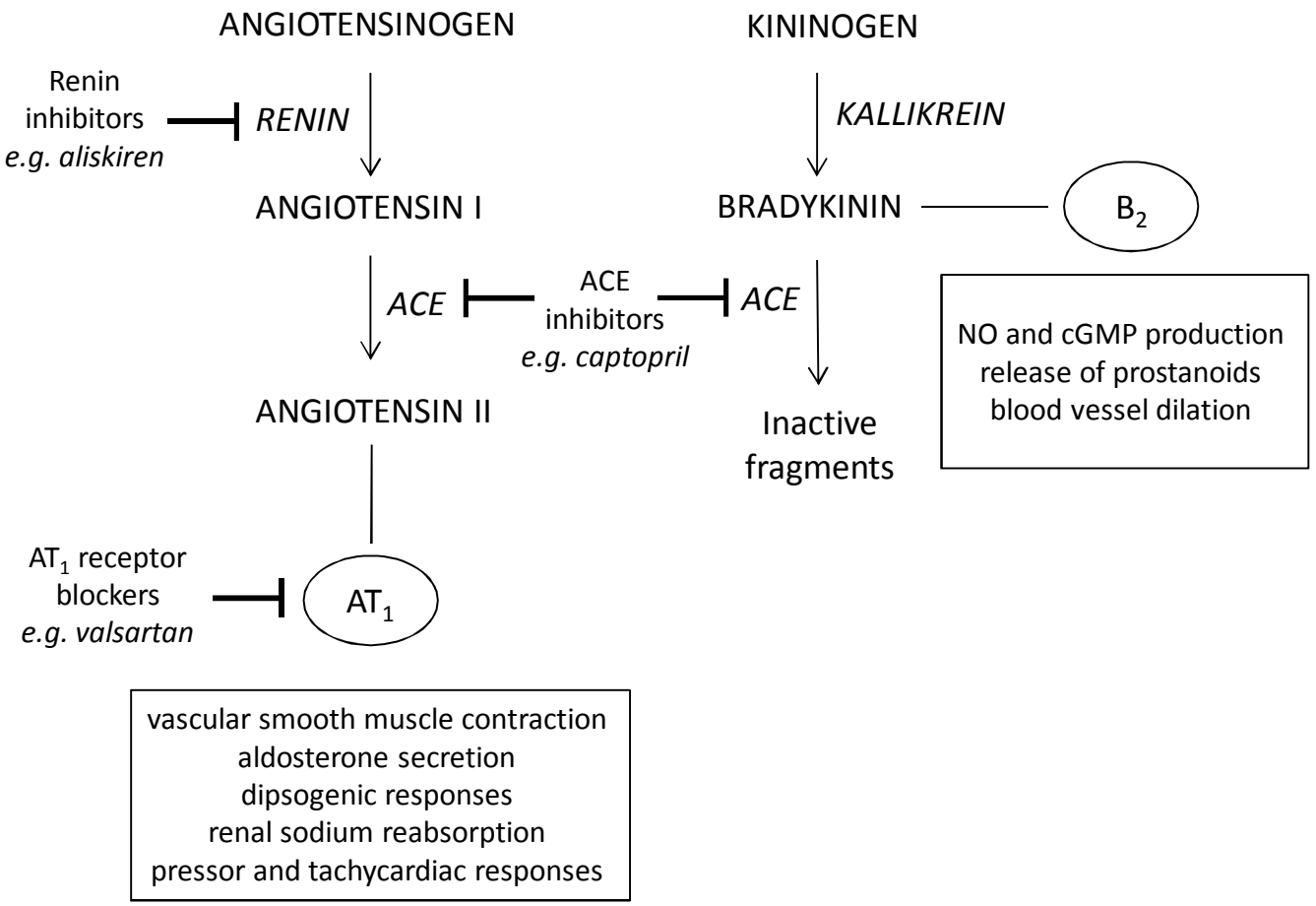

Figure 2 Manzanares et al 2015

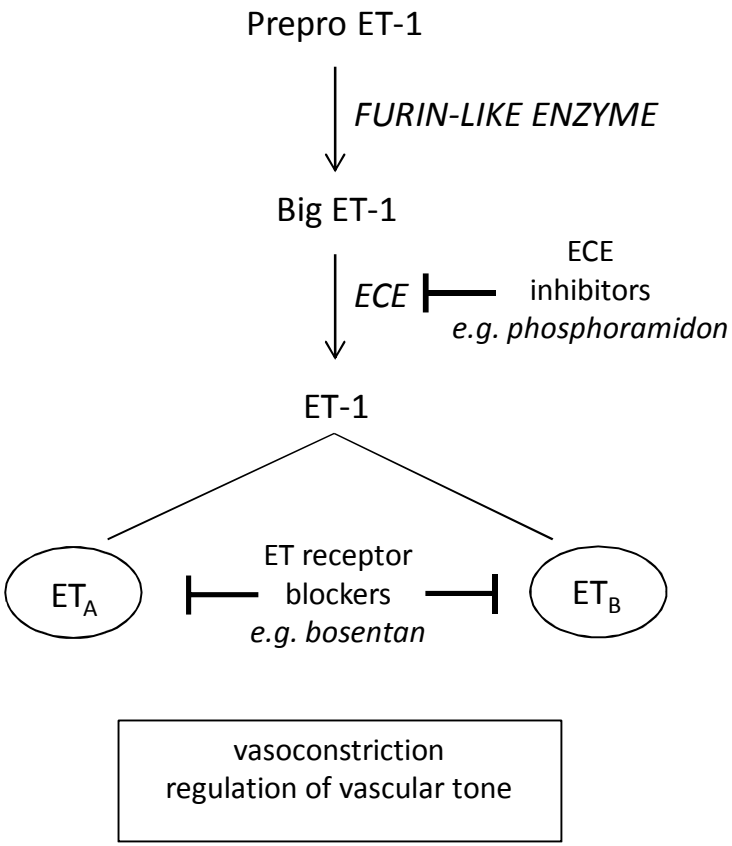

Figure 3 Manzanares et al 2015

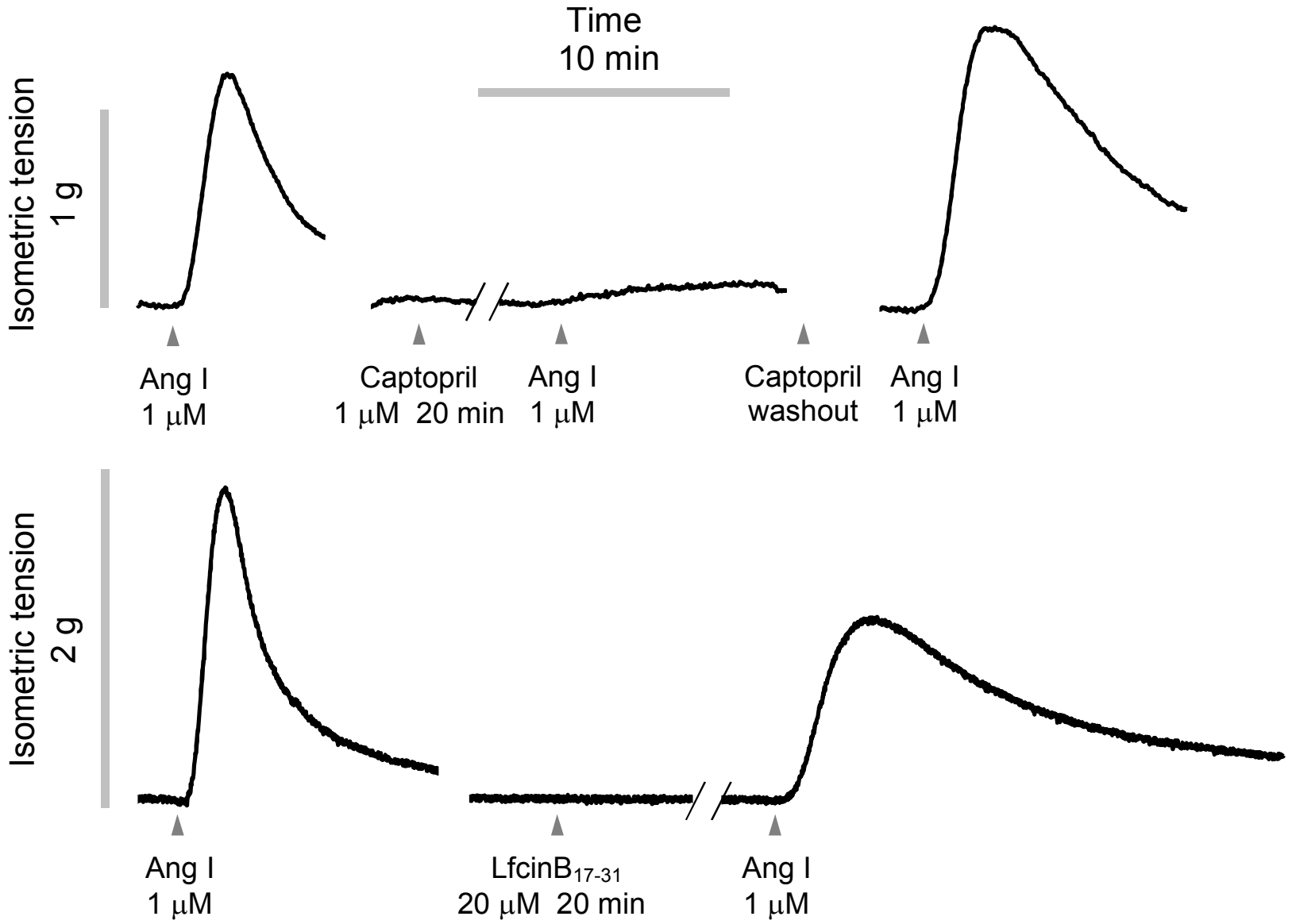

Figure 4 Manzanares et al 2015