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

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**Abstract**

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

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

**Abbreviations**

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

## 1. Introduction

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

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

Despite numerous efforts, the *in vivo* mechanism underlying vasoactive and blood pressure lowering effects of antihypertensive food-derived peptides has not yet been fully established. Beyond *in vivo* ACE inhibition reported for some peptides and hydrolysates (Yang, Yang, Chen, Tzeng, & Han, 2004; Lu et al., 2011; Wang et al., 2012; García-Tejedor et al., 2014), antihypertensive effects can be mediated via other components of the RAAS system, such as renin which

catalyzes the cleavage of the N-terminal region of angiotensinogen to release angiotensin I (Udenigwe, Lin, Hou, & Aluko, 2009; Fitzgerald et al., 2012; Ajibola, Fashakin, Fagbemi, & Aluko, 2013). Moreover, emerging evidence points to the arginine–nitric oxide pathway, the endothelin system or opioid receptors as molecular targets for food-derived antihypertensive peptides (Udenigwe & Mohan, 2014).

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

Endothelial cells line the internal surface of blood vessels, play critical roles in vascular biology and represent a good model to evaluate the molecular mechanisms involved in blood pressure regulation. Since the effects of antihypertensive peptides might result from synergic interactions with several targets, global approaches represent a feasible strategy for revealing the action of these peptides through distinct pathways. With the aim of characterizing the effect of LFH at the cellular level, its inhibitory effect on ACE activity in cultured human endothelial cells was determined. Moreover, the effects of LFH on the expression of a panel of genes related to hypertension were evaluated in these cells. Finally, in the light of mRNA expression results, the effects of LFH on the NO pathway, including NO production, have been specifically assessed.

## 2. Materials and methods

### 2.1. Materials

Bovine LF was provided by FrieslandCampina Domo (Zwolle, The Netherlands). Bicinchoninic acid protein assay kit, porcine pepsin, gelatin, monoclonal anti-β-Actin antibody and trypan blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). ACE substrate o-aminobenzoyl-Gly-p-nitro-Phe-Pro was provided by Bachem Feinchemikalien (Bubendorf, Switzerland). Primary human umbilical vein endothelial cells (HUVEC) pooled from two to four different umbilical cords (Advancell, Barcelona, Spain) were a kind gift of Dr. Isabel Fariñas (Department of Cellular Biology, Universidad de Valencia, Spain). EndoGRO-VEGF complete medium kit was provided by Millipore (Darmstadt, Germany). Phosphate buffered saline solution (PBS) and trypsin-EDTA solution were obtained from HyClone (Logan, UT, USA). RNeasy Mini Kit, $RT^2$ First Strand Kit and Human

Hypertension RT² Profiler™ PCR Array from SABiosciences were purchased from Qiagen (Valencia, CA, USA). LightCycler® 480 SYBR Green I Master and Complete Mini protease-inhibitor cocktail were provided by Roche Diagnostics (Basel, Switzerland). Protran BA 85 nitrocellulose, horseradish peroxidase-linked species-specific secondary antibodies and ECL Select™ Western Blotting Detection Reagent were purchased from GE Healthcare (Buckinghamshire, United Kingdom). Polyclonal anti-mNOSTRIN antibody was a kind gift of Dr. Stephanie Oess (Goethe Universität, Frankfurt am Main, Germany).

2.2. Bovine Lactoferrin Enzymatic Hydrolysate (LFH) and Synthetic Peptides

Bovine lactoferrin was hydrolyzed using porcine pepsin and the product was subjected to ultrafiltration through a polyethersulfone membrane with a 3 kDa cut-off (Vivascience, Sartorius Stedim Biotech, Aubagne, France) as previously described (Ruiz-Giménez et al., 2012). The permeate (LFH) was kept at -20ºC until use.

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

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

2.3. Cell Culture

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

Cells from passages 3 to 5 were washed with PBS solution, detached with trypsin-EDTA solution, resuspended in complete medium and evaluated for viability by microscopic observation of cells stained with 0.2 % trypan blue. HUVEC were seeded onto gelatin-coated 6-well plates at a cellular density of 25.000 viable cells/$cm^2$. Twenty four hours after seeding, the corresponding treatment was added directly to the cell culture supernatant, and maintained in contact with the cells for additional 24 hours. The treatments were used at the following final concentrations: LFH 700 µg/mL; RPYL and LIWKL, 100 µM. At the end of the experiments cell viability was determined by the trypan blue exclusion method. No loss of viability was observed after any of the treatments.

2.4. ACE Activity Measurements

Three different biological replicates of either the control (non-treated cells), or LFH-treated cells, were evaluated for ACE activity present in cell culture supernatants. Activity was measured in all the replicates just before treatment (zero time) and at 1, 3, 6 and 24h after addition of LFH. ACE activity was measured using the fluorescent method described by Sentandreu & Toldrá (2006) with some modifications. Fifty µL samples were taken at every time point along the experiment, and transferred to a 96-well black plate. Two hundred µL of the internally quenched fluorescent substrate o-aminobenzoyl-Gly-p-nitro-Phe-

Pro (0.45 mM) were added and the production of the fluorescent product by ACE activity was recorded (excitation 320 nm, emission 405 nm) every 30 minutes for at least 4 hours using a Fluoroskan Ascent FL (ThemoFisher, Waltham, MA ). Relative ACE activity in treated cells was referred to controls at the same time points.

2.5. RNA Isolation and Real-time Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) Analysis

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

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

(SABiosciences, Qiagen). Four different biological replicates for each control or treatment condition were assayed for mRNAs quantitation.

Further quantification of mRNA levels was carried out for those genes whose expression ratio obtained using the PCR array was modified with statistical significance by a factor of 2 or greater. For that purpose, specific oligonucleotide pairs used as PCR primers were selected for guanylate cyclase 1, soluble, alpha 3 (GUCY1A3; forward: 5'-GTCCTGCAGTGTACCACGAA-3', reverse: 5'-GGCAGATTCCGGGGATTTCA-3'; product length: 140 bp), nitric oxide synthase trafficking (NOSTRIN; forward: 5'-CTCAACCCAGCCATCCTTGT-3', reverse: 5'-GATTGCTCTGCCCACCAGAA-3'; product length: 147 bp), and prostaglandin-endoperoxide synthase 2 (PTGS2; forward: 5'-CGACTCCCTTGGGTGTCAAA-3', reverse: 5'-AAGTGCTGGGCAAAGAATGC-3'; product length: 136 bp); and also for the housekeeping genes selected, namely β-actin (ACTB; forward: 5'-AGCGAGCATCCCCCAAAGTTCAC-3', reverse: 5'-GGGTGGCTTTTAGGATGGCAAGG-3'; product length: 127 bp), and ribosomal protein L13a (RPL13A; forward: 5'-TGCCCCACAAAACCAAGCGAGG-3', reverse: 5'-AGGCTTCAGACGCACGACCTTG-3'; product length: 131 bp). In those cases, 3.5 µg of total RNA were retro-transcribed using $RT^2$ First Strand Kit in a final volume of 111 µL. Two µL of that dilution was used as template in a 10 µL PCR reaction using the LightCycler® 480 SYBR green I master. Primers were used at a final concentration of 250 nM and efficiency was determined for every primer pair at this final concentration using a pooled cDNA as template. The $C_T$ data were obtained by the LighCycler® 480 software (release 1.5.0) and were analyzed by the $\Delta\Delta C_T$ method using the Relative Expression Software Tool

version 2.0.13 (Qiagen). Three different biological replicates for each control or treatment condition were assayed for specific mRNAs quantitation.

### 2.6. Quantitation of NOSTRIN Protein Levels by Western Blot

Flow-through from the RNA-purification column was precipitated with 4 volumes of cold acetone, let stay on ice for 30 min and centrifuged at maximum speed in a microcentrifuge for 10 min. The pellet was resuspended in buffer (50 mM Tris, 10 mM NaCl, 2 mM EDTA, 1% v/v Igepal CA 630, 5% v/v glycerol, 1mM phenylmethylsulfonyl fluoride) containing Complete Mini protease-inhibitor cocktail. Equivalent amounts of the total protein extracts were separated by SDS-PAGE and blotted onto Protran BA 85 nitrocellulose. The membranes were probed using polyclonal anti-mNOSTRIN antibody which recognizes human NOSTRIN with an apparent molecular mass slightly under 60 kDa and also using monoclonal anti-β-Actin antibody (Sigma-Aldrich). Horseradish peroxidase-linked species-specific were used as secondary antibodies. The probed membranes were developed by chemiluminiscence with ECL Select™ Western Blotting Detection Reagent using a LAS-1000 instrument (Fujifilm, Tokyo, Japan). Densitometric analysis of the bands was performed using ImageJ 1.48v software (NIH, Bethesda, MD, USA).

### 2.7. Nitric Oxide Content

Nitric oxide generation was estimated in cell culture supernatants by quantitation of its stable product nitrite using colorimetric determination with the Griess reagent (Tsikas, 2007). In brief, cell culture supernatants were deproteinized with zinc sulphate (final concentration 1.5% w/v; 5 min on ice), centrifuged at

maximum speed for 15 minutes in a microcentrifuge, and equal volumes of the clear supernatant and of the Griess reagent incubated at room temperature for 15 minutes. Absorbance at 540 nm was recorded for blanks and samples. A standard curve for nitrite was obtained using concentrations between 0 and 1.25 μM of sodium nitrite dissolved in complete HUVEC medium.

### 2.8. Statistics

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

## 3. Results

### 3.1. Effect of LFH on ACE activity

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

Table 1 shows ACE residual activity in HUVEC incubated for 24 h with LFH (700 μg/mL) with respect to non-treated cells (control). LFH induced a significant

inhibition of ACE activity. After 1 h of treatment, when maximum inhibitory effect was observed, ACE residual activity in LFH-treated cells was 15.3 ± 0.4 %.The inhibitory effect remained significant up to 24 h ($P < 0.05$; Student's *t* test) and it was higher than 60 %.

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

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

Out of the 84 genes included in the array, expression of 51 genes could be detected in the conditions tested. LFH treatment caused statistically significant upregulation of 4 genes (ARG2, GUCY1A3, GUCY1B3 and PTGS2) and downregulation of 2 genes (ATP6AP2 and NOSTRIN). Genes whose expression was modified by a factor of 2 or greater, were GUCY1A3 (4.42-fold) and PTGS2 (2.23-fold) that were upregulated, and NOSTRIN (2.45-fold) that was downregulated. GUCY1A3 which encodes guanylate cyclase 1 $\alpha$3 subunit and NOSTRIN which encodes NO synthase trafficking are related to NO generation, whereas PTGS2 (also called COX2) which encodes prostaglandin-endoperoxide synthase 2 or cyclooxygenase 2 is a key component of the prostaglandin synthesis. Based on their expression profiles, these three genes were considered for further studies.

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

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

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

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

NOSTRIN is a regulatory protein which modulates NO production (Zimmermann et al., 2002), and NO-mediated vasodilation has been attributed to some food-

derived peptides (Boelsma & Kloek, 2009; García-Redondo, Roque, Miguel, López-Fandiño, & Salaices, 2010). This prompted us to further study NOSTRIN as a target for LFH and to investigate whether the reduction of NOSTRIN mRNA levels detected after LFH treatment resulted in any change in protein expression and in NO production.

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

## 4. Discussion

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

Inhibition of ACE activity by LFH at a concentration of 700 μg/mL was shown in cultured HUVEC supernatants. LFH-inhibitory action on ACE was comparable to that reported for two casein trypsin hydrolyates which showed $IC_{50}$ values in the μg/mL level (Rousseau-Ralliard et al., 2010). Despite inhibition of ACE activity at 24 h, LFH did not affect ACE mRNA levels at the same time point. Although transcriptional regulation of ACE at earlier time points cannot be discarded, our results are in agreement with previous works that describe no significant changes in expression of genes associated with RAAS in aorta of SHR after repeated administration of the well-known ACE-inhibitory peptides VPP and IPP were described (Yamaguchi, Kawaguchi, & Yamamoto, 2009). In HUVEC, VPP and IPP had no effect on ACE protein expression, although ACE mRNA levels were not evaluated (Hirota et al., 2011). By contrast, downregulation of the mRNA levels of renin, ACE and AT1 was reported in kidney from SHRs after repeated treatment with the ACE-inhibitory sequences IQP and RVPSL (Lu et al., 2011; Yu, Yin, Zhao, Chen, & Liu, 2014). Despite ECE has also been described as a target for LFH (Fernández-Musoles et al., 2013b), and similarly to what we describe here for ACE gene expression, we did not find significant changes in ECE relative mRNA level or in expression of other genes associated with the endothelin system (EDN1, EDN2, EDNRA, EDNRB, see Table 2). Time-course effect of LFH in HUVEC on mRNA levels of genes associated with RAAS and endothelin system are necessary to discard any regulation at the mRNA level at shorter exposure times. Regulation via post-transcriptional or post-translational mechanisms such as mRNA stability and translation and protein-protein interactions cannot be ruled out (Chattopadhyay et al., 2005; Kohlstedt et al., 2013).

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

ACE inhibitors are considered to act protectively on endothelium by inhibiting production of angiotensin II and degradation of bradykinin, which facilitates NO production. NO is an endogenous vasodilatory gaseous molecule that continuously regulates the diameter of blood vessels and maintains an anti-proliferative and anti-apoptopic environment in the vessel wall, thus contributing to protect the endothelium (Sessa, 2004). The action of NO as a vasodilator is mediated by the activation of vascular smooth muscle soluble guanylate cyclase (sGC), the physiological receptor for NO (Arnold, Mittal, Katsuki, & Murad, 1977)

and presumably the most relevant molecular target for NO-releasing drugs in human cardiovascular therapy (Zabel, Weeger, La, & Schmidt, 1998). sGC is a signal transduction enzyme that forms the second messenger molecule cyclic GMP which in turn modulates the activity of several effector proteins that lead to vasorelaxation (Schmidt, Lohmann, & Walter, 1993). Our results showed increased NO production and upregulated expression of sGC (GUCY1) following LFH treatment in HUVEC, suggesting an elevation of cyclic GMP in HUVEC, as described for vasodilator compounds which *in vivo* activate sGC in vascular tissue (Galle et al., 1999).

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

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

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

In contrast to that found for LFH, the sequences LIWKL and RPYL did not elicit any significant change in the level of expression of PTGS2/COX-2,

GUCY1A3 or NOSTRIN genes. These results might reveal a negligible contribution of both peptides to the effects on mRNA levels provoked by LFH treatment, at least at the time point tested, and suggest that other peptides of the hydrolysate could contribute to such effect on gene expression in HUVEC. Synergistic effects of individual peptides cannot be discarded as suggested for the stimulatory effect on mucin gene expression in human intestinal cells of a casein hydrolysate and derived peptides (Martínez-Maqueda, Miralles, Cruz-Huerta, & Recio, 2013). Finally, stability of LFH and peptides in HUVEC during treatment has not been addressed here, and potential degradation by the action of cellular peptidases including ACE should be considered. Undoubtedly the possible effects of individual sequences contained in LFH as well as bioavailability issues merit further research.

## 5. Conclusions

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

of LFH. Results from the PCR array serve as a ground point for future studies on the complex mechanism of action underlying the antihypertensive effects of ACE-inhibitory peptides derived from food proteins, including lactoferrin-derived peptides.

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**Figure captions**

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

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

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

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

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

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

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

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

| | | |
|---|---|---|
| GUCY1B3 | Guanylate cyclase 1, soluble, beta 3 | **1.81*** |
| HIF1A | Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor) | -1.13 |
| ITPR1 | Inositol 1,4,5-trisphosphate receptor, type 1 | -1.09 |
| ITPR2 | Inositol 1,4,5-trisphosphate receptor, type 2 | -1.10 |
| KCNMA1 | Potassium large conductance calcium-activated channel, subfamily M, alpha member 1 | -1.17 |
| MYLK | Myosin light chain kinase | 1.21 |
| MYLK2 | Myosin light chain kinase 2 | -1.19 |
| NOS3 | Nitric oxide synthase 3 (endothelial cell) | 1.17 |
| NOSIP | Nitric oxide synthase interacting protein | 1.20 |
| NOSTRIN | Nitric oxide synthase trafficking | **-2.45**** |
| NPR1 | Natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A) | -1.56 |
| P2RX4 | Purinergic receptor P2X, ligand-gated ion channel, 4 | 1.22 |
| PDE3A | Phosphodiesterase 3A, cGMP-inhibited | -1.20 |
| PDE3B | Phosphodiesterase 3B, cGMP-inhibited | -1.27 |
| PDE5A | Phosphodiesterase 5A, cGMP-specific | 1.11 |
| PLCG1 | Phospholipase C, gamma 1 | -1.31 |
| PLCG2 | Phospholipase C, gamma 2 (phosphatidylinositol-specific) | -1.13 |
| PRKG1 | Protein kinase, cGMP-dependent, type I | 1.03 |
| PTGIR | Prostaglandin I2 (prostacyclin) receptor (IP) | 1.22 |
| PTGS1 | Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase) | 1.28 |
| PTGS2 | Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) | **2.23*** |
| S1PR1 | Sphingosine-1-phosphate receptor 1 | -1.19 |
| SCNN1A | Sodium channel, nonvoltage-gated 1 alpha | -1.79 |
| SLC7A1 | Solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 | 1.04 |
| SPHK1 | Sphingosine kinase 1 | -1.39 |
| SPHK2 | Sphingosine kinase 2 | -1.03 |

[a]Final concentration in the assay 700 µg/mL.

[b]Fold change was calculated by the ΔΔCT method . *significantly different from control $P < 0.05$; ** significantly different from control $P < 0.01$ (Student's *t*-test; n=4).

Figure 1 García-Tejedor et al., 2014

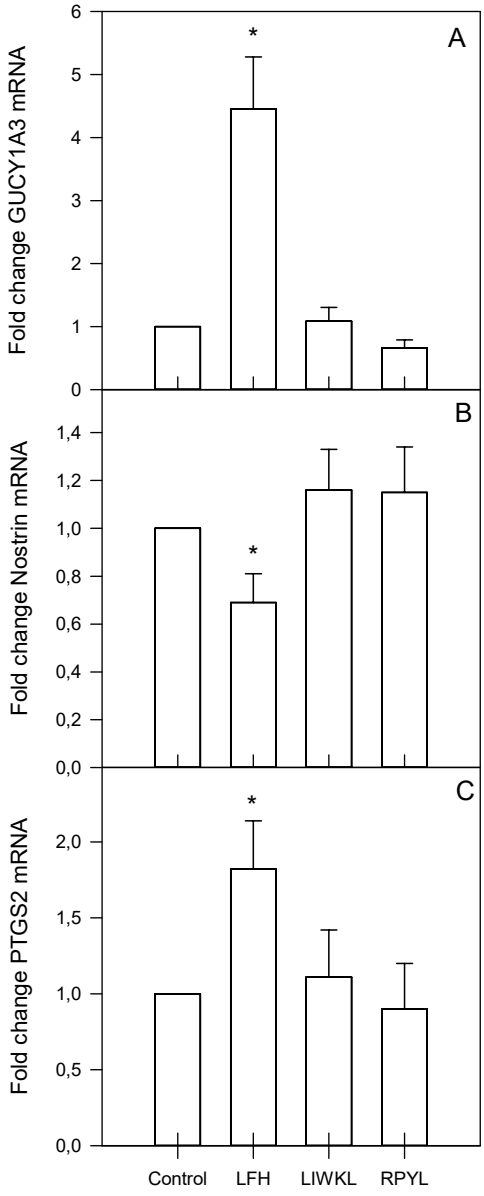

Figure 2 García-Tejedor et al 2014

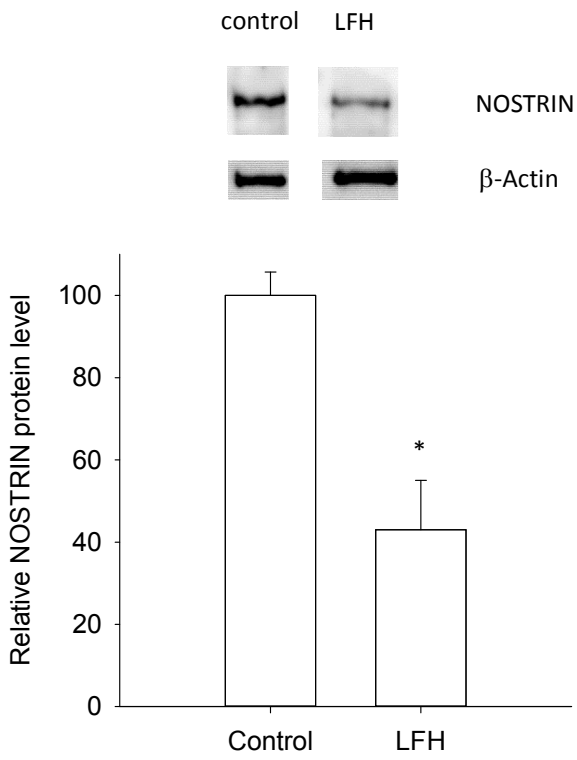

Figure 3 García-Tejedor et al., 2014