1 **Vasoactive properties of antihypertensive lactoferrin-derived peptides in resistance vessels:**
2 **effects in small mesenteric arteries from SHR rats**

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Abstract

Aims: Bovine lactoferrin (LF) hydrolysates and peptides identified thereof have shown antihypertensive effects in rat models, mainly but not exclusively by angiotensin-converting enzyme inhibition. In this study we aimed to assess the vasoactive effects and mechanisms of an ultrafiltered (<3 kDa) pepsin LF hydrolysate (LFH) and a heptapeptide identified in a LF hydrolysate produced by yeast proteolysis (DPYKLRP) in peripheral resistance arteries from spontaneously hypertensive rats (SHRs).

Main methods: We used a myograph system for isometric tension recording in isolated small mesenteric arteries from SHRs. Direct vasoactive effects of LFH (30-100 µg/mL) and DPYKLRP (30-100 µM) were assessed in arteries precontracted with phenylephrine (PE, 10 µM) or KCl (120 mM), and in PE-precontracted arteries preincubated (10 min) with the NO synthase inhibitor L-NAME (0.1 mM) or the cyclooxygenase inhibitor indomethacin (10 µM). Indirect vasoactive effects of LFH (30-100 µg/mL) or DPYKLRP (30-100 µM) preincubation (10 min) on the relaxant responses to the NO donor sodium nitroprusside (SNP, 0.01-10 µM) or acetylcholine (Ach, 1-100 µM) were also studied in PE-precontracted arteries.

Key findings: Both LHF and DPYKLRP elicited direct relaxation of mesenteric arteries, by a mechanism involving NO release, counteracting modulation by prostanoids and K⁺ efflux. Moreover, LF-derived peptides also showed indirect vasoactive effects by enhancing endothelium-dependent relaxation to Ach and endothelium-independent relaxation to SNP.

Significance: In conclusion, LF-derived peptides show *ex vivo* direct and indirect relaxing effects in small mesenteric arteries from SHRs. These vasoactive effects would reduce vascular peripheral resistance *in vivo*, and thus contribute to their antihypertensive effects.

Keywords: Lactoferrin-derived peptides; Spontaneously hypertensive rat; Mesenteric artery; Vasorelaxation; Antihypertensive effects; Action mechanism

1. Introduction

Hypertension is the biggest single contributor to the global burden of disease and to global mortality. The numbers of people affected and the prevalence of high blood pressure worldwide are expected to increase over the next decade. Preventive strategies are therefore urgently needed, especially in less developed countries, and management of hypertension must be optimized [1]. Pharmacological treatment for hypertension is effective in reducing both blood pressure and morbimortality from cardiovascular and renal diseases. However, long-term pharmacological therapy can have adverse effects and requires continuous medical supervision [2].

Lifestyle changes, also named nonpharmacological therapy, safely and effectively delay or prevent hypertension in non-hypertensives, delay or prevent drug therapy in hypertensives, and contribute to blood pressure reduction in hypertensives already on drug therapy [3]. Increased physical activity, reduced salt intake, weight loss, moderation of alcohol intake, smoking cessation, increased potassium intake, and an overall healthy dietary pattern, termed the Dietary Approaches to Stop Hypertension (DASH) diet, effectively lower blood pressure [4,5].

Antihypertensive DASH-type dietary patterns are rich in fruits and vegetables, include whole grains, poultry, fish, nuts and low-fat dairy products, and are reduced in red meat, sweets, and sugar-containing beverages. These aliments are rich in potassium, magnesium, calcium, dietary fibre and protein, and have reduced fat (total and saturated) and cholesterol. It is likely that several aspects of the diet, rather than just one nutrient or food, account for blood pressure reduction [6,7].

Many antihypertensive constituents have been characterized from different food sources [8]. Among these bioactive compounds, research in the past 20 years has shown that food protein-derived peptides, released during food processing or gastrointestinal digestion, represent a suitable group of natural compounds that could serve as alternative antihypertensive agents on peptide-formulated functional foods or nutraceuticals [9]. Some of these peptides have been shown to act on different molecular targets involved in the pathophysiology of hypertension, and exhibited potent *in vivo* antihypertensive activity in both animal models and human clinical trials [10].

Consumption of milk and low-fat dairy products has been inversely related to hypertension in both epidemiological and interventional studies like DASH trial. Although casein and whey have been shown to decrease blood pressure also as such, research has been focused on their degradation products, peptides liberated from their parent protein by enzymatic hydrolysis during gastrointestinal digestion, fermentation of milk with proteolytic starter cultures or hydrolysis by exogenous enzymes [11].

The milk protein lactoferrin (LF), which possesses a diverse range of physiological functions such as antimicrobial/antiviral, immunomodulatory and antioxidant activities [12], was more than a decade ago pointed out in *in silico* studies as a promising source of angiotensin-converting enzyme (ACE)-inhibitory peptides [13]. Since then, our group has focused on the characterization of the antihypertensive effects and molecular targets of LF-derived peptides using different experimental approaches and models. Beyond ACE inhibition, the mechanisms of action underlying the blood pressure-lowering effects of LF-derived peptides include their interaction with different components of the renin-angiotensin (RAS) and endothelin (ET) systems as well as expression modulation of genes encoding proteins involved in the nitric oxide (NO) pathway and prostaglandin synthesis [14].

LFH is an ultrafiltered (<3 kDa) bovine LF hydrolysate obtained by pepsin digestion, with antihypertensive effects in spontaneously hypertensive rats (SHRs) after oral acute [15] and long-term administration [16]. DPYKLRP is a LF-derived heptapeptide (protein fragment f[70–76]) isolated and identified in a protein ultrafiltered (<3 kDa) hydrolysate produced by the proteolytic yeast *Kluyveromyces marxianus*. DPYKLRP also showed antihypertensive effect when orally administered to SHRs, including *in vivo* ACE inhibition and dose-dependent reductions in blood pressure of magnitude and duration similar to those of the antihypertensive drug captopril [17].

To gain further insight into the antihypertensive mechanisms of LF-derived peptides, in the present study we aimed to know the direct and indirect vasoactive effects of both LFH and DPYKLRP, as well as the involvement of K⁺ efflux, NO and prostanoids. For this purpose, we used isolated small mesenteric arteries from SHRs, which are resistance vessels determining peripheral resistance and thus systemic blood pressure.

2. Material and methods

2.1 Lactoferrin-derived peptides, drugs and solutions

Bovine LF was provided by FrieslandCampina Domo (Zwolle, The Netherlands). LF was hydrolysed using porcine pepsin (Sigma-Aldrich, Madrid, Spain) 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 [15]. Protein content of the permeate LFH was estimated by the bicinchoninic acid method using bovine serum albumin (Sigma-Aldrich) as standard. LFH was freeze-dried and kept at -20 °C until use. LF-derived peptide of sequence DPYKLRP [17] was purchased at >95% purity from GenScript Corporation (Piscataway, NJ, USA), wherein it was synthesized by solid phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry. Heptapeptide concentration was based on the dry weight.

Acetylcholine chloride (Ach), indomethacin (IND), N ω -nitro-L-arginine methyl ester hydrochloride (L-NAME), L-phenylephrine hydrochloride (PE) and sodium nitroprusside dihydrate (SNP) were obtained from Sigma-Aldrich.

The Ringer-Locke solution had the following composition (mM): NaCl 120, KCl 5.4, CaCl₂ 2.2, MgCl₂ 1.0, NaHCO₃ 25 and glucose 5.6. In 120 mM KCl-depolarizing solution, NaCl was replaced by an equimolar amount of KCl.

2.2 Animals and ethical issues

Eight nine-week-old male spontaneously hypertensive rats (SHRs) weighing 200-250 g (Charles River Laboratories, Barcelona, Spain) were housed in temperature-controlled rooms (23°C) with 12 h light/dark cycles, and consumed tap water and standard diets *ad libitum*. A two week period of acclimatization was allowed to recover from the stress associated with transportation [18]. Experiments were conducted in compliance with the legislation on protection of animals used for scientific purposes in Spain (RD 53/2013) and the EU (Directive 2010/63/EU). Protocols were approved by the Animal Experimentation Ethics Committee from IIS La Fe.

Indirect measurement of systolic and diastolic arterial blood pressure was carried out in restrained awake rats by the non-invasive tail cuff method [17] using computer-assisted Non-Invasive Blood Pressure equipment (LE5001 unit with LE5160R cuff and transducer, Panlab Harvard Apparatus, Barcelona, Spain). Before the measurements, rats were kept at 37°C during 15 min to make the pulsations of the tail artery detectable. Each blood pressure value was obtained by averaging at least three consecutive and successful measurements without disturbance of the signal.

The SHR_s used in the present study ($BW = 262 \pm 3$ g; $n = 8$) showed consistently elevated values of both systolic blood pressure ($SBP = 182 \pm 2$ mm Hg) and diastolic blood pressure ($DBP = 117 \pm 3$ mm Hg).

2.3 Isolation of mesenteric arteries

Rats were euthanized in a CO₂ chamber, followed by decapitation and exsanguination. A mid-line laparotomy was carried out to exteriorize the mesenteric bed. Intestine (10 cm) with its feeding vasculature, including part of the superior mesenteric artery, was removed and pinned in a dissecting Petri dish with black Sylgard® bottom containing physiological saline. Vascular arcades were excised and arteries isolated by removing the mesenteric membrane, the vein and connective tissue.

2.4 Isometric tension recording in small mesenteric arteries

Segments (length ≈ 2 mm) of second- and third-order small mesenteric arteries (inner diameter < 250 μ M) were mounted in a Multi Wire Myograph System (Model 610M, Danish Myo Technology, Aarhus, Denmark) for isometric tension recording by means of PowerLab 8/30 data acquisition hardware and Chart 5 software (ADInstruments, Castle Hill, Australia). The myograph baths contained 7 mL of Ringer-Locke solution at 37°C, bubbled with a 95% O₂ and 5% CO₂ mixture to give a pH of 7.3-7.4.

To optimize active arterial responses, passive tension of each arterial segment was normalized by stepwise distension to an internal circumference $IC_1 = 0.9 IC_{100}$, where IC_{100} was the internal circumference that the fully relaxed vessel would have at 100 mm Hg transmural pressure [19]. To reactivate the vessels and check for functionality, they were started by repeated 120 mM KCl stimulation until maximal contractions were obtained. Finally, to check for endothelial function, the arterial segments were precontracted with PE (10 μ M) and relaxed with Ach (10 μ M).

Direct vasoactive effects of LFH (30-100 $\mu\text{g/mL}$) and sequence DPYKLRP (30-100 μM) were assessed in arterial segments precontracted with either PE (10 μM) or KCl (120 mM). The involvement of NO and prostanoids in the responses to LFH and DPYKLRP in PE-precontracted arteries was assessed by preincubation (10 min) with either the NO synthase (NOS) inhibitor L-NAME (0.1 mM) or the cyclooxygenase (COX) inhibitor IND (10 μM), respectively. Indirect vasoactive effects of LFH (30-100 $\mu\text{g/mL}$) and sequence DPYKLRP (30-100 μM) were studied by assessing the effect of preincubation (10 min) with either the hydrolysate or the individual peptide on relaxant responses to either the NO donor SNP (0.01-10 μM) or Ach (1-100 μM) in PE-precontracted arteries.

2.5 Data analysis and statistics

Blood pressure values were expressed in mm Hg and data were mean $\pm$ SEM from 'n' rats. Absolute vasoconstriction values were expressed in mN, whereas relative vasorelaxation values were expressed in % from precontraction. Data were mean $\pm$ SEM from 'n' arterial segments obtained from different rats. Statistical comparison of mean values was carried out by unpaired Student's t-test or one-way ANOVA followed by Dunnett test for multiple comparisons, using GraphPad InStat® software. Differences were considered significant when $P < 0.05$.

3. Results

3.1 Lactoferrin hydrolysate and peptide DPYKLRP induced relaxation of SHR mesenteric artery

In SHR small mesenteric artery segments (1.60 ± 0.05 mm long, $n = 26$), high- K^+ solution (KCl 120 mM) induced rapid phasic contraction followed by tonic contraction eliciting an active tone of 6.17 ± 0.54 mN (i.e. 3.91 ± 0.36 mN/mm). On the other hand, phenylephrine (PE, 10 μM) also induced phasic contraction, followed by tonic contraction up to 7.70 ± 0.73 mN of active tone. Acetylcholine (Ach, 10 μM) induced rapid loss of the PE-induced active

tone, with a maximal relaxation of 5.13 ± 0.74 mN ($n = 23$). Fig. 1 shows typical recordings, representative of these contractile and relaxant responses in SHR small mesenteric artery.

LFH (30-100 μ g/mL) induced significantly concentration-dependent relaxation of PE-precontracted mesenteric artery ($P < 0.05$), with a maximal relaxation of 55.1 ± 3.7 % of the active tone ($n = 18$) (Fig. 2A). KCl-precontracted artery was also relaxed in a significantly concentration-dependent manner by LFH ($P < 0.05$), but in contrast to PE-precontracted artery, maximal relaxation was 6.6 ± 1.4 % of the active tone ($n = 18$) (Fig. 2A).

The individual peptide DPYKLRP (30-100 μ M) elicited significantly concentration-dependent relaxation of PE-precontracted mesenteric artery ($P < 0.01$), with a maximal relaxation of 50.6 ± 2.8 % of the active tone ($n = 16$) (Fig. 2B). By contrast, maximal relaxation induced by DPYKLRP in KCl-precontracted artery was 11.4 ± 2.3 % of the active tone ($n = 18$) (Fig. 2B).

3.2 NO and prostanoids mediated relaxation of SHR mesenteric artery induced by lactoferrin-derived peptides

Concentration-dependent relaxation induced by LFH in PE-precontracted mesenteric artery (Fig. 3A) was significantly inhibited by preincubation with the NO synthase (NOS) inhibitor L-NAME (0.1 mM) ($P < 0.01$). By contrast, LFH-elicited relaxation was significantly enhanced by preincubation with the cyclooxygenase (COX) inhibitor indomethacin (IND, 10 μ M) ($P < 0.01$).

With regard to the sequence DPYKLRP (Fig. 3B), concentration-dependent relaxation was significantly inhibited by preincubation with L-NAME (0.1 mM) ($P < 0.01$). Mesenteric artery preincubated with IND (10 μ M) showed slightly increased DPYKLRP-induced relaxation, but did not reach statistical significance ($P > 0.05$).

3.3 Lactoferrin-derived peptides enhanced NO-induced relaxation of SHR mesenteric artery

Sodium nitroprusside (SNP, 0.01-10 μ M) induced concentration-dependent relaxation of PE-precontracted mesenteric artery, with a maximal relaxation of 86.3 ± 7.7 % of the active tone (n = 12) (Fig. 4). Preincubation with LFH (30 μ g/mL) significantly increased the relaxant response to SNP ($P < 0.01$), except for the maximal SNP concentration (Fig. 4A). On the other hand, preincubation with the individual peptide DPYKLRP (30 μ M) significantly increased the relaxant response to SNP ($P < 0.05$ or $P < 0.01$, depending on SNP concentration), except for the maximal SNP concentration (Fig. 4B).

Acetylcholine (Ach, 1-100 μ M) elicited concentration-dependent relaxation of PE-precontracted mesenteric artery, with a maximal relaxation of 64.1 ± 4.2 % of the active tone (n= 23) (Fig. 5). Preincubation with LFH (100 μ g/mL) (Fig. 5A) or the heptapeptide (100 μ M) (Fig. 5B) produced slight but significant increases in the relaxant responses to some of the Ach concentrations ($P < 0.05$).

4. Discussion

In the present study, we have shown that direct and indirect vasoactive effects of both LFH and DPYKLRP in the small mesenteric artery contribute to vascular relaxation and, by reducing vascular peripheral resistance, would be involved in their antihypertensive effects in SHRs. Most of food-derived antihypertensive peptides target the ACE activity, but emerging evidence has pointed to other antihypertensive mechanisms beyond ACE inhibition [14,20,21], as demonstrated here for LF-derived peptides.

Our present results show that both LFH and the peptide DPYKLRP relax the distal mesenteric artery of hypertensive rats. The well-known casein-derived tripeptides, VPP and IPP, induced relaxation of Wistar rat aorta [22], but few studies have previously reported relaxant effects of milk protein derived peptides in the mesenteric vascular bed. Camel and

bovine casein tryptic hydrolysates showed vasorelaxant effect in mesenteric artery of Wistar Kyoto rats [23]. With regard to individual peptides, casomokinin L (YPFPPL), a derivative of casoxin D (YVPFPPF) originally isolated from a human casein hydrolysate, showed vasorelaxing activity on the canine mesenteric artery [24]. We for the first time report direct relaxant effects of milk protein derived peptides in small distal mesenteric arteries from hypertensive rats, which have a predominant role in the regulation of blood pressure through the control of the systemic vascular resistance. Our findings are in line with relaxant effects of different original or modified egg protein derived peptides in canine mesenteric artery [25], hypertensive rat mesenteric artery [26,27], and small distal branches of rat mesenteric artery [28].

Both LFH and the peptide DPYKLRP relax the mesenteric artery precontracted with the adrenergic receptor agonist PE and, to a much lower extent, relax arterial segments precontracted by strong depolarization with high- K^+ . Activity of membrane K^+ channels in blood vessel smooth muscle contributes to determination of membrane potential and vascular tone. The electrochemical gradient for K^+ ions is such that opening of K^+ channels results in diffusion of this cation out of the cells, membrane hyperpolarization and vascular relaxation [29]. Decreasing the K^+ gradient across the cell membrane by raising extracellular K^+ reduces K^+ efflux, which suggests that inhibition of relaxation induced by LF-derived peptides in high- K^+ conditions takes place because the peptides relax the rat mesenteric artery by increasing K^+ channel conductance and subsequent K^+ efflux.

LFH- and DPYKLRP-elicited relaxations were strongly reduced by the NO synthase (NOS) inhibitor L-NAME, suggesting that release of the endothelium derived relaxing factor NO is involved in the relaxant effect of LF-derived peptides. Endothelium-dependent relaxations to Ach indicated the presence of functionally competent endothelium in the mesenteric arteries used in this study. Indeed, we have previously shown that LFH enhanced NO production in

cultured endothelial cells [30]. NO relaxes the rat mesenteric artery by inducing smooth muscle hyperpolarization via the NO-cGMP-PKG pathway [31] or by directly activating K^+ channels [32]. According to our results in high K^+ -precontracted arteries, LF-derived peptides could induce the release of NO, which in turn would stimulate K^+ efflux to elicit vasorelaxation. In line with our results, a NO-dependent mechanism has also been reported for mesenteric artery relaxations induced by camel and bovine casein tryptic hydrolysates [23] as well as for relaxation to casomokinin L, a modified casein-derived peptide [24].

Our results also showed that LFH- and to a lower extent DPYKLRP-elicited relaxations were enhanced by the COX inhibitor indomethacin, thus supporting a role for vasoconstrictor prostanoids in LF-induced relaxations. Apart from NO, both locally produced vasodilator and vasoconstrictor prostanoids modulate vascular tone [33], and the involvement of vasoconstrictor prostanoids has been reported in PE-induced vasoconstriction of the rat mesenteric artery [34]. Thus, our results suggest that LF-induced relaxations are mediated by NO release but partially counteracted by a net vasoconstrictor effect of prostanoids. In contrast with the present study, no effect of COX inhibition was observed on the relaxation of mesenteric arteries elicited by casein hydrolysates [23] or casomokinin L [24]. It should be noted that proximal rat and canine mesenteric arteries were used in those studies. Quite interestingly, COX inhibition produced increase instead of reduction of mesenteric artery relaxation induced by different original or modified egg protein derived peptides [25,27,28], thus indicating the involvement of prostanoids with a net relaxant contribution. Therefore, the role of COX products in mesenteric artery relaxation seems to depend on the peptide sequence/source, the proximal or distal artery level and the animal species, as well as on its normotensive or hypertensive state.

Endothelium-dependent relaxation induced by Ach in the rat mesenteric resistance arteries is mediated by NO release. By contrast, the NO donor DEA-NO elicits endothelium-

independent relaxations [35]. Hypertension and aging induce endothelial dysfunction, thus impairing endothelium-dependent relaxations, as reported for Ach-induced relaxation in the mesenteric artery of SHR [36] and old Wistar rats [37]. Some previous studies assessed the effects of food-derived antihypertensive peptides, by means of chronic *in vivo* intake (weeks) or long-time *ex vivo* incubation (hours), on relaxation of mesenteric arteries from hypertensive rats. Thus, long-term intake (8 weeks) of fermented milk products containing the casein-derived bioactive tripeptides IPP and VPP by salt-loaded Goto-Kakizaki hypertensive rats preserved Ach-induced endothelium-dependent relaxations of the superior mesenteric artery, while endothelium-independent relaxations elicited by the NO donor SNP were not modified [38]. Similarly, long-term (18 days) oral treatment of SHR with antihypertensive egg white protein ovotransferrin-derived tripeptides (IRW, IQW and LKP) preserved NO-dependent relaxation to methacholine in branches of the mesenteric artery [39,40], as did long-term (12 days) oral treatment with the whole egg white hydrolysate [41]. With regard to *ex vivo* exposure to peptides, twelve hour storage of superior mesenteric arteries from SHR with IPP and VPP preserved Ach-induced relaxations, while relaxations to SNP did not change [42]. Similarly, the vasodilatory effects of both Ang-(1-7) and bradykinin in superior mesenteric arteries from SHR were enhanced after six hour incubation with IPP [43].

In the present study with small SHR mesenteric arteries, a relative impairment of endothelium-dependent relaxation to Ach (~65%), when compared to almost full relaxation to SNP (~85%) was observed. Short-term (minutes) preincubation with either LFH or the peptide DPYKLRP enhanced SNP-induced and, to a lower extent, Ach-induced relaxation. These results point to an effect of LF-derived peptides on vascular signal transduction downstream NO release by Ach, but elucidation of the mechanism deserves further research. In line with our results, the tetrapeptides α -lactorphin (YGLF), from milk α -lactalbumin, and β -lactorphin (YLLF), from β -lactoglobulin, improved the relaxant response to Ach in the superior mesenteric artery from SHR, whereas only β -lactorphin improved the relaxation to SNP [36]. On the other

hand, casein-derived IPP improved bradykinin-induced vasorelaxation (independently from ACE inhibitory effects preventing bradykinin degradation) in the superior mesenteric artery from normotensive old (aged 22 wk) Wistar rats [37]. Altogether, these results support an indirect vasoactive role for LF-derived and other milk-derived peptides in acute improvement of both endothelium-dependent and endothelium-independent relaxation of mesenteric arteries.

5. Conclusion

Milk-protein LF-derived peptides with proven antihypertensive effects induce direct relaxation of small mesenteric arteries from SHR, by a mechanism involving NO release, counteracting modulation by prostanoids and K⁺ efflux. Moreover, LF-derived peptides also show indirect vasoactive effects by enhancing both Ach- and NO-elicited relaxation. Altogether, these vasoactive effects would reduce vascular peripheral resistance and thus contribute to their antihypertensive effects in SHR.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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Figure captions

Fig. 1 - Functionality of isolated segments of small mesenteric artery from spontaneously hypertensive rat for isometric tension myography. **(A)** Representative recording of the contractile response to arterial smooth muscle depolarization by high- K^+ (KCl, 120 mM). The small inflection is an artefact due to aspiration by the bath emptying pump. **(B)** Representative recording of the contractile responses to adrenergic receptor stimulation by increasing concentrations of phenylephrine (1-10 μ M), followed by relaxant response to endothelial cholinergic receptor stimulation by acetylcholine (10 μ M).

Fig. 2 - Direct relaxant effects of lactoferrin (LF)-derived peptides in small mesenteric artery from spontaneously hypertensive rat. **(A)** Concentration-dependent relaxation to LF hydrolysate (LFH, 30-100 μ g/mL) in arterial segments precontracted by phenylephrine (10 μ M) or high- K^+ (KCl, 120 mM). **(B)** Concentration-dependent relaxation to the individual peptide DPYKLRP (30-100 μ M) in arterial segments precontracted by phenylephrine (10 μ M) or high- K^+ (KCl, 120 mM). Data are mean $\pm$ SEM from 16-18 arterial segments. * $P < 0.05$, ** $P < 0.01$.

Fig. 3 – Effects of NO synthase (NOS) and cyclooxygenase (COX) inhibition on the relaxant effects of lactoferrin (LF)-derived peptides in small mesenteric artery from spontaneously hypertensive rat. **(A)** Concentration-dependent relaxation to LF hydrolysate (LFH, 30-100 μ g/mL) in arterial segments precontracted by phenylephrine (10 μ M), in the absence (control) and in the presence of the NOS inhibitor L-NAME (0.1 mM) or the COX inhibitor indomethacin (10 μ M). **(B)** Concentration-dependent relaxation to the individual peptide DPYKLRP (30-100 μ M) in arterial segments precontracted by phenylephrine (10 μ M), in the absence (control) and in the presence of L-NAME (0.1 mM) or indomethacin (10 μ M). Data are mean $\pm$ SEM from 14-18 arterial segments. ** $P < 0.01$.

Fig. 4 - Indirect vasoactive effects of lactoferrin (LF)-derived peptides by improving endothelium-independent relaxant responses in small mesenteric artery from spontaneously

hypertensive rat. **(A)** Concentration-dependent relaxation to the NO donor sodium nitroprusside (0.01-10 μ M) in arterial segments precontracted by phenylephrine (10 μ M), in the absence (control) and in the presence of LF hydrolysate (LFH, 30 μ g/mL). **(B)** Concentration-dependent relaxation to sodium nitroprusside (0.01-10 μ M) in arterial segments precontracted by phenylephrine (10 μ M), in the absence (control) and in the presence of the individual peptide DPYKLRP (30 μ M). Data are mean $\pm$ SEM from 10-12 arterial segments. *P < 0.05, **P < 0.01.

Fig. 5 - Indirect vasoactive effects of lactoferrin (LF)-derived peptides by improving endothelium-dependent relaxant responses in small mesenteric artery from spontaneously hypertensive rat. **(A)** Concentration-dependent relaxation to acetylcholine (1-100 μ M) in arterial segments precontracted by phenylephrine (10 μ M), in the absence (control) and in the presence of LF hydrolysate (LFH, 30-100 μ g/mL). **(B)** Concentration-dependent relaxation to acetylcholine (1-100 μ M) in arterial segments precontracted by phenylephrine (10 μ M), in the absence (control) and in the presence of the individual peptide DPYKLRP (30-100 μ M). Data are mean $\pm$ SEM from 12-23 arterial segments. *P < 0.05.

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