Food derived peptides reduced COX-2 expression in angiotensin ii-stimulated adventitial fibroblasts

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Abstract: Prostanoids modulate the pathogenesis of vascular diseases such as atherosclerosis, in which inflammation has an important role. It is well known that inducible Ciclooxygenase-2 (COX-2) is responsible for prostanoid production associated with inflammation. Angiotensin II may be implicated through the expression of COX-2 in the vascular wall. The purpose of this study was to examine in angiotensin II-stimulated adventitial fibroblasts the anti-inflammatory activity of different food peptides by inhibiting COX-2 expression, and the production of pro-inflammatory prostanoids. Fibroblasts from aorta of Sprague-Dawley rats were incubated with different food-derived peptides followed by incubation with Angiotensin II. COX-2 expression was determined by western blot, transcriptional activity by luciferase assays and prostaglandin E2 by enzyme immunoassay. COX-2 expression was inhibited in the presence of Val-Pro-Pro (bovine β-casein 84–86), Arg-Asp-Ile-Leu-Asn-Gln (ovalbumin 84–89) and Tyr-Arg-Gly-Gly-Leu-Glu-Pro-Ile-Asn-Phe (ovalbumin 125–134). Angiotensin II-induced prostaglandin E2 production was also reduced by all the above-mentioned sequences. The incubation with ovalbumin-derived peptides displayed a significant reduction of COX-2 promoter activity compared to the stimuli with Angiotensin II in transiently transfected cells. These three sequences could potentially be used as functional food ingredients to reduce inflammation related to cardiovascular diseases.

Keywords: Bioactive peptides, hypertension, ciclooxygenase-2, fibroblast, functional food.

1. Introduction

Over the last years, ample data has demonstrated the pivotal role of low-grade inflammation in the pathophysiology of atherosclerosis and cardiovascular diseases. Considering hypertension, the inflammatory process is implicated in this pathophysiology through a bidirectional relationship since arterial hypertension may enhance inflammation and vice versa [1]. The renin-angiotensin system (RAS) is classically known as a regulator of blood pressure, cardiac function and fluid homeostasis [2].
Recent evidence also indicates a critical role for RAS inhibition in obesity and Type 2 Diabetes [3]. Angiotensin II (Ang II), an important vasoactive peptide that physiologically regulates vascular tone, is one of the main factors involved in hypertension-induced tissue damage. Ang II has been implicated in hypertension through its significant pro-inflammatory actions in the vascular wall [4]. This molecule promotes the induction of inflammatory enzymes such as COX-2 [5-7] and mPGES-1 [8-10] in vascular and nonvascular cells. It is known that vascular adventitia is a critical regulator of vessel wall function in health and disease conditions and, in particular, it is known that Ang II increases COX-2 expression and prostanooid production in adventitial fibroblasts [7].

In recent years, it has been recognized that many dietary constituents may contribute to human cardiovascular health [11]. Dietary proteins have shown to offer health benefits in vitro and/or in vivo either as intact proteins or partially digested, called bioactive peptides. Various studies have highlighted the possibility of using food-derived compounds as natural products to control the metabolic complications related to Metabolic Syndrome, including insulin resistance [3, 12], glucose homeostasis alterations [13] and beneficial properties on the lipid profile [14].

Among them, bioactive food peptides with antihypertensive properties have been the most extensively studied of all the bioactivities induced by food protein hydrolysates [15]. Our research group has previously reported the antihypertensive properties of egg-derived peptides after short [16] and long-term administration to spontaneously hypertensive rats [17]. Their antihypertensive effect has been related to angiotensin converting enzyme (ACE) inhibition, antioxidant and vasodilator mechanisms [18,19].

Bearing in mind the information above mentioned, the reduction of vascular inflammation could be also implicated in the antihypertensive mechanisms of food-derived peptides. Therefore, the purpose of this study was to examine the vascular anti-inflammatory activity of several antihypertensive egg- and milk-derived peptides in angiotensin II-stimulated adventitial fibroblasts from aorta of Sprague-Dawley (SD) rats.

2. Materials and Methods

Drugs and Synthetic Peptides.

Angiotensin II was purchased from Sigma Chemical, Co. (St. Louis, MO, USA). The different egg-derived peptides used were previously identified in egg white hydrolysed with pepsin and characterized as antihypertensive (FRADHPFL, RADHPFL, RADHPF, RDILNQ, YAEERYPIL, YRGGLEPINF, ESIINF, IVF) [16, 18, 20], and also sequences characterized after the simulated gastrointestinal digestions (RADHP, YPI, YR, NF) [17]. Furthermore, the anti-inflammatory activity of the antihypertensive peptides IPP and VPP derived from fermented milk was also tested [21, 22]. All these peptides were obtained by conventional Fmoc solid phase synthesis and they were synthesized and provided by GenScript Corporation (Piscataway, NJ, 08854, USA), and their purity (>90%) was verified in our laboratory by RP-HPLC-MS/MS (Agilent technologies, Santa Clara, CA, USA).

Animals.

Six-month-old male SD rats were obtained from colonies maintained at the Animal Quarters of the faculty of medicine (Autónoma University of Madrid). Before the experimental period, rats were euthanized by decapitation and thoracic aortas were isolated and processed to obtain primary cultures of adventitial fibroblasts. The investigation was performed in accordance with the European and Spanish legislation on care and use of experimental animals (210/63/UE; Real Decreto 53/2013, respectively).

Cell culture.

Primary cultures of aortic fibroblasts were obtained according to a previously described method [23]. Briefly, thoracic aortas from SD rats were aseptically removed, cleaned from fat tissue and blood cells, and placed in DMEM/F-12 (HAM) medium (Sigma Chemical, Co., St. Louis, MO,
USA) (4ºC) containing 0.1% bovine serum albumin (BSA), 100 U/ml of penicillin, 100 µg/ml of streptomycin. The aortas were digested in the same medium containing 2 mg/ml of collagenase (type II, Worthington, Lakewood, USA), and incubated for 30 min at 37ºC in a humidified atmosphere of CO2 (5%). Then, the adventitia was peeled from medial smooth muscle and endothelial layers using forceps, cut into small pieces and placed on 60x15 mm tissue dishes in DMEM/ F-12 (HAM) medium (Sigma Chemical) supplemented with 10% fetal calf serum (Biological Industries, Kibbutz, Israel), containing 100 U/ml of penicillin, 100 µg/ml of streptomycin (Gibco, Invitrogen, Paisley, UK). Cells were allowed to reach confluence (6-8 days).

Confluent cells were passaged with PBS/trypsin-EDTA (Sigma Chemical) and the resulting cell suspension was washed by centrifugation. Then, the cells were resuspended in DMEM/ F-12 (HAM) medium containing 20 ng/ml of basic fibroblast growth factor (bFGF) (Invitrogen, Carlsbad, USA) and 5 µg/ml of heparin (Sigma Chemical) and seeded at a density of 30%, as described (Masur et al., 1996). Cells were identified as fibroblasts by morphological and growth characteristics, by the lack of immunocytochemical staining with specific monoclonal anti-α-actin antibody (Sigma Chemical) and by positive staining with specific monoclonal anti-vimentin antibody (NeoMarkers, Westinghouse, CA, USA). Cells from identically treated lines were used between passages 2 and 5.

**Experimental protocol.**

Aortic fibroblasts were cultured into 6-well plates until 80% of confluence. The day before the experiments, the medium was replaced with fresh fasting medium (DMEM / F-12 (HAM) medium containing 0.1% BSA (Sigma Chemical), 100 U/ml of penicillin and 100 µg/ml of streptomycin). The culture medium was refreshed just before treatment. For the experiments aimed to analyze the COX-2 expression, quiescent cells were incubated with vehicle (control) or angiotensin II (0.1 µM) for 4 h. The effects of several peptide sequences on the protein expression induced by 4 h incubation with angiotensin II were analysed. All peptides were added to the cells at 100 µM 1 hour before adding Ang II.

**Western blot analysis.**

After the treatment, aortic fibroblasts were washed twice in ice-cold PBS buffer (GIBCO, California, USA). The cells were scraped and whole-cell lysates were prepared in the homogenization buffer: 1 mM sodium vanadate, 1% sodium dodecyl sulfate (SDS) and 10 mM Tris-HCl, pH 7.4. Protein content of whole cell lysates was determined with Lowry protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA), using bovine serum albumin as standard. Total protein equivalents of each sample (20 µg) were separated on a 7 % SDS-polyacrylamide gel and electrophoretically transferred to polyvinylidene difluoride membranes (Amersham, GE Healthcare, Buckinghamshire, UK) in Tris-Glycine transfer buffer with 20% methanol in a Bio-Rad Trans-Blot Cell (Bio-Rad). Membranes were blocked with 5 % skimmed milk and 5 % BSA (Sigma Chemical), in TBS-Tween for two hours at room temperature before incubation with antibodies for COX-2 (1:200; Cayman Chemical, Ann Arbor, MI, USA), COX-1 (1:500; Cayman Chemical) and vimentin (1:200; NeoMarkers) overnight at 4ºC. Membranes were thoroughly washed and incubated with horseradish peroxidase-coupled anti-rabbit (1:2,000; BioRad) or anti-mouse (1:5,000; Stressgen, Victoria, Canada) immunoglobulin G antibodies to detect COX-2 and COX-1 or vimentin respectively, for 1 h at room temperature and finally washed four times in TBS-Tween. Bands were detected using the ECL plus Western Blotting detection system (GE Healthcare) after exposure to X-Ray AX film (Konica Minolta, Tokio, Japan). Signals on the immunoblot were quantified using a computer program (NIH Image V1.56). Vimentin expression was used as loading control.

**PGE2, measurements.**

Cells were grown and harvested as described above. Cells were then incubated for 24 h with vehicle (control) or with Ang II (0.1 µM) for 4 h in the absence or in the presence of different peptides. The culture media was collected and release of PGE2 was determined using the
Prostaglandin E2, ELISA Kit according to the manufacturer’s instructions (Cayman Chemical). PGE2 concentration was corrected by the protein content of each sample well.

**Transient transfection assays.**

Transient transfections of aortic fibroblasts were performed with LipofectamineTM LTX Reagent (Invitrogen) and the luciferase reporter vector containing 3.9 kb of COX-2 promoter (pGL3-COX-2P) that includes a region from 3074 to 3236 of human COX-2 mRNA cloned in the XbaI site of the pGL3 basic vector, kindly provided by Dr. W. Eberhardt (Klinikum der Johann Wolfgang Goethe-Universität Frankfurt am Main, Germany) [24], with the pCMV-βgal (Clontech, Japan) as internal control vector. Cells were seeded on twelve-well plates (80,000 cells/well) and transient transfection assays were performed with 1 µg plasmid/well, 0.25 µg/well of pCMV-β-gal, 2 µL of LipofectamineTM LTX Reagent and 1 µL Plus reagent following manufacturer’s instructions. The complexes DNA/liposome were added to the cells for 18 h. Then cells were incubated overnight in serum free medium and finally stimulated with angiotensin II for 24 hours. Luciferase activity was measured in cell lysates using the Luciferase assay kit (BioThema AB, Handen, Sweden) and a luminometer (Orion II, Berthold detection systems) according to the manufacturer’s instructions. Results were normalized by β-galactosidase activity using the Enzyme Assay System (BioThema AB).

**Statistical analysis.**

Data are presented as mean ± standard error (± SE). n indicates the number of experiments. Statistical analysis was done by Student’s t test or by one or two-way ANOVA followed by a Bonferroni test. Values were considered to be significant when P < 0.05.

### 3. Results and Discussion

The effect of the different peptide sequences derived from food proteins on inflammation was examined using fibroblasts stimulated with Ang II as a model for testing vascular anti-inflammatory activity of these peptides. Fibroblasts were treated with Ang II (0.1 µM for 4 h), and Western Blot analysis was performed to assess the influence of various food-derived peptides on COX-2 expression. COX-2 expression was scarce in non-stimulated adventitial fibroblasts; it was significantly increased in fibroblasts after 4 h of incubation with Ang II. The expression levels of COX-1 were similar in non- and stimulated cells and no differences were observed in COX-1 expression in Ang II stimulated fibroblasts.

All peptides studied were ineffective in COX-2 expression in the absence of Ang II. After 4 h of incubation with Ang II, COX-2 expression was reduced by several peptides, but only significantly by three sequences (VPP, RDILNQ and YRRGGLEPINF), and the most efficient reduction of COX-2 expression was in response to YRRGGLEPINF (Figure 1). The results of our study provide first evidence for the vascular anti-inflammatory activity of egg-derived peptides.
Figure 1. Western Blot analysis of ciclooxygenase 2 (COX-2) expression in fibroblasts from aorta of Sprague-Dawley rats, previously incubated with different antihypertensive food peptides (3-12; 100 mM; 1 hour), followed by incubation with Angiotensin II (0.1 mM; 4 hours). Vimentin and Ciclooxygenase 1 (COX-1) expression were used as loading controls. Immunoblots were quantified using the software NIH Image v1.56. Values are means ± SE (n = 4)* P<0.05 Angiotensin II vs control cells, #P<0.05 Food derived peptides vs Angiotensin II cells.

To determine whether the effect of these peptides in Ang II-induced COX-2 expression was due to changes in transcription rate, COX-2 transcriptional activity was measured in fibroblasts transiently transfected with a reporter plasmid containing 3900 bp of the human COX-2 promoter fused to luciferase cDNA. Ang II significantly increased the transcriptional activity of the COX-2 promoter while the pre-incubation with VPP, RDILNQ or YRGGLEPINF one hour prior to the addition of stimuli, reduced significantly and differentially the luciferase activity. In Ang II-triggered cells, the luciferase activity was nearly reduced to basal levels after incubation with RDILNQ or YRGGLEPINF but not in the presence of VPP (Figure 2).
Figure 2. Luciferase activity (RLU) measured from the transcriptional activation of Ciclooxygenase-2 promoter (pGL3-COX-2P) in aortic fibroblast from Sprague-Dawley rats, by angiotensin II (Ang II) and different food derived peptides (VPP, RDILNQ and YRGGLEPINF). Results were normalized by β-galactosidase activity. Values are means ± SE (n = 4)* P<0.05 Angiotensin II vs control, #P<0.05 Food derived peptides vs Angiotensin II.

It has been traditionally recognized that inducible COX-2 is responsible for prostanoid production associated with inflammation [25], and COX-1 is important for the prostanoids with "housekeeping" functions, such as gastric cytoprotection [26]. There is evidence that COX-2 is upregulated in a several number of inflammatory diseases and COX-2 inhibitors have beneficial effects in inflammatory diseases [27]. However, selective COX-2 inhibitors increase cardiovascular risk probably by decreasing the protective production of endothelial PGI2. Non-steroidal anti-inflammatory drugs that inhibit COX-2 expression, among other effects, are used to treat acute inflammation, but these compounds are unsuccessful in the treatment of chronic inflammatory diseases, such as rheumatoid arthritis or osteoarthritis. Furthermore, these compounds exhibit several undesired side effects [28]. Therefore, alternative treatments, with safer compounds, are needed. Previously, Yamaguchi et al. in 2009 demonstrated that the bovine-milk derived α-lactalbumin inhibited the COX-2 activity in induced inflammation animal models [29]. Moreover, the food protein derived peptides are considered to be milder, safer and easily absorbed compared with other synthetic drugs [30]. Therefore, the results suggest that RDILNQ or YRGGLEPINF could be a safe and useful natural drug for patients with severe pain that requires anti-inflammatory drugs.

In parallel with the Western Blot assays, the concentration of PGE2 released in the cell supernatants was determined. PGE2 production increased after Ang II incubation and it was significantly reduced by the peptides sequences VPP, RDILNQ and YRGGLEPINF (Figure 3).

Figure 3. Prostaglandin E2 (PGE2) determination in the supernatants of aortic fibroblast from Sprague-Dawley rats, previously incubated with or without Angiotensin II (Ang II; 0.1 mM; 24 hours), and different food derived peptides (VPP, RDILNQ and YRGGLEPINF). Prostaglandin E2 production was measured by ELISA. Values are means ± SE (n = 4)* P<0.05 Angiotensin II vs control, #P<0.05 Food derived peptides vs Angiotensin II.

PGE2 production has been related to cardiovascular diseases and this molecule is considered one of the most atherogenic prostanoids, mediating progression of atherogenesis through several
mechanisms, such as induction of other pro-inflammatory mediators and adhesion molecules, facilitating migration of macrophages and other immune cells [7].

4. Conclusions

Based on the positive results obtained in this study related to COX-2 expression and PGE2 levels, several food-derived peptides tested in this study could potentially be used as functional food ingredients with the purpose of providing anti-inflammatory effects. These food-derived peptides could contribute to the reduction and prevention of inflammation and related diseases. The results observed could also improve the additional value of eggs as a routine and cheap source of numerous biologically active compounds and could be use as functional food ingredient to reduce inflammatory processes related to vascular damage in hypertension.

Author contributions

Conception and design of the study: M.G.-R., M.G., M.S., M.M. Acquisition of data: M.G.-R., M.G., M.M. Analysis and interpretation of data: M.G.-R., M.G., M.S., M.M. Drafting the article: M.G.-R., M.M. Revising the article and approval of the final version to be submitted: M.G.-R., M.G., M.S., M.M.

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Conflicts of interest

The authors declare no conflict of interest.

References


