Article

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].

46 Recent evidence also indicates a critical role for RAS inhibition in obesity and Type 2 Diabetes
47 [3]. Angiotensin II (Ang II), an important vasoactive peptide that physiologically regulates vascular
48 tone, is one of the main factors involved in hypertension-induced tissue damage. Ang II has been
49 implicated in hypertension through its significant pro-inflammatory actions in the vascular wall [4].
50 This molecule promotes the induction of inflammatory enzymes such as COX-2 [5-7] and mPGES-1
51 [8-10] in vascular and nonvascular cells. It is known that vascular adventitia is a critical regulator of
52 vessel wall function in health and disease conditions and, in particular, it is known that Ang II
53 increases COX-2 expression and prostanoid production in adventitial fibroblasts [7].

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

60 Among them, bioactive food peptides with antihypertensive properties have been the most
61 extensively studied of all the bioactivities induced by food protein hydrolysates [15]. Our research
62 group has previously reported the antihypertensive properties of egg-derived peptides after short
63 [16] and long-term administration to spontaneously hypertensive rats [17]. Their antihypertensive
64 effect has been related to angiotensin converting enzyme (ACE) inhibition, antioxidant and
65 vasodilator mechanisms [18,19].
66 Bearing in mind the information above mentioned, the reduction of vascular inflammation
67 could be also implicated in the antihypertensive mechanisms of food-derived peptides. Therefore,
68 the purpose of this study was to examine the vascular anti-inflammatory activity of several
69 antihypertensive egg- and milk-derived peptides in angiotensin II-stimulated adventitial fibroblasts
70 from aorta of Sprague-Dawley (SD) rats.

71 **2. Materials and Methods**

72 *Drugs and Synthetic Peptides.*
73 Angiotensin II was purchased from Sigma Chemical, Co. (St. Louis, MO, USA). The different
74 egg-derived peptides used were previously identified in egg white hydrolysed with pepsin and
75 characterized as antihypertensive (FRADHPFL, RADHPFL, RADHPF, RDILNQ, YAEERYPIL,
76 YRGGLEPINF, ESIINF, IVF) [16, 18, 20], and also sequences characterized after the simulated
77 gastrointestinal digestions (RADHP, YPI, YR, NF) [17]. Furthermore, the anti-inflammatory activity
78 of the antihypertensive peptides IPP and VPP derived from fermented milk was also tested [21, 22].
79 All these peptides were obtained by conventional Fmoc solid phase synthesis and they were
80 synthesized and provided by GenScript Corporation (Piscataway, NJ, 08854, USA), and their purity
81 (>90%) was verified in our laboratory by RP-HPLC-MS/MS (Agilent technologies, Santa Clara, CA,
82 USA).

83

84 *Animals.*
85 Six-month-old male SD rats were obtained from colonies maintained at the Animal Quarters of
86 the faculty of medicine (Autónoma University of Madrid). Before the experimental period, rats were
87 euthanized by decapitation and thoracic aortas were isolated and processed to obtain primary
88 cultures of adventitial fibroblasts. The investigation was performed in accordance with the
89 European and Spanish legislation on care and use of experimental animals (210/63/UE; Real Decreto
90 53/2013, respectively).

91

92 *Cell culture.*
93 Primary cultures of aortic fibroblasts were obtained according to a previously described
94 method [23]. Briefly, thoracic aortas from SD rats were aseptically removed, cleaned from fat tissue
95 and blood cells, and placed in DMEM/F-12 (HAM) medium (Sigma Chemical, Co., St. Louis, MO,

96   USA) (4ºC) containing 0.1% bovine serum albumin (BSA), 100 U/ml of penicillin, 100 µg/ml of
97   streptomycin. The aortas were digested in the same medium containing 2 mg/ml of collagenase
98   (type II, Worthington, Lakewood, USA), and incubated for 30 min at 37ºC in a humidified
99   atmosphere of CO2 (5%). Then, the adventitia was peeled from medial smooth muscle and
100 endothelial layers using forceps, cut into small pieces and placed on 60x15 mm tissue dishes in
101 DMEM/ F-12 (HAM) medium (Sigma Chemical) supplemented with 10% fetal calf serum (Biological
102 Industries, Kibbutz, Israel), containing 100 U/ml of penicillin, 100 µg/ml of streptomycin (Gibco,
103 Invitrogen, Paisley, UK). Cells were allowed to reach confluence (6-8 days).
104 Confluent cells were passaged with PBS/trypsin-EDTA (Sigma Chemical) and the resulting cell
105 suspension was washed by centrifugation. Then, the cells were resuspended in DMEM/ F-12 (HAM)
106 medium containing 20 ng/ml of basic fibroblast growth factor (bFGF) (Invitrogen, Carlsbad, USA)
107 and 5 µg/ml of heparin (Sigma Chemical) and seeded at a density of 30%, as described (Masur et al.,
108 1996). Cells were identified as fibroblasts by morphological and growth characteristics, by the lack of
109 immunocytochemical staining with specific monoclonal anti-α-actin antibody (Sigma Chemical) and
110 by positive staining with specific monoclonal anti-vimentin antibody (NeoMarkers, Westinghouse,
111 CA, USA). Cells from identically treated lines were used between passages 2 and 5.
112
113 *Experimental protocol.*
114 Aortic fibroblasts were cultured into 6-well plates until 80% of confluence. The day before the
115 experiments, the medium was replaced with fresh fasting medium (DMEM / F-12 (HAM) medium
116 containing 0.1% BSA (Sigma Chemical), 100 U/ml of penicillin and 100 µg/ml of streptomycin). The
117 culture medium was refreshed just before treatment. For the experiments aimed to analyze the
118 COX-2 expression, quiescent cells were incubated with vehicle (control) or angiotensin II (0.1 µM)
119 for 4 h. The effects of several peptide sequences on the protein expression induced by 4 h incubation
120 with angiotensin II were analysed. All peptides were added to the cells at 100 µM 1 hour before
121 adding Ang II.
122
123 *Western blot analysis.*
124 After the treatment, aortic fibroblasts were washed twice in ice-cold PBS buffer (GIBCO,
125 California, USA). The cells were scraped and whole-cell lysates were prepared in the
126 homogenization buffer: 1 mM sodium vanadate, 1% sodium dodecyl sulfate (SDS) and 10 mM
127 Tris-HCl, pH 7.4. Protein content of whole cell lysates was determined with Lowry protein assay
128 reagent (Bio-Rad Laboratories, Hercules, CA, USA), using bovine serum albumin as standard. Total
129 protein equivalents of each sample (20 µg) were separated on a 7 % SDS-polyacrylamide gel and
130 electrophoretically transferred to polyvinylidene difluoride membranes (Amersham, GE Healthcare,
131 Buckinghamshire, UK) in Tris-Glycine transfer buffer with 20% methanol in a Bio-Rad Trans-Blot
132 Cell (Bio-Rad). Membranes were blocked with 5 % skimmed milk and 5 % BSA (Sigma Chemical), in
133 TBS-Tween for two hours at room temperature before incubation with antibodies for COX-2 (1:200;
134 Cayman Chemical, Ann Arbor, MI, USA), COX-1 (1:500; Cayman Chemical) and vimentin (1:200;
135 NeoMarkers) overnight at 4ºC. Membranes were thoroughly washed and incubated with
136 horseradish peroxidase-coupled anti-rabbit (1:2,000; BioRad) or anti-mouse (1:5,000; Stressgen,
137 Victoria, Canada) immunoglobulin G antibodies to detect COX-2 and COX-1 or vimentin
138 respectively, for 1 h at room temperature and finally washed four times in TBS-Tween. Bands were
139 detected using the ECL plus Western Blotting detection system (GE Healthcare) after exposure to
140 X-Ray AX film (Konica Minolta, Tokio, Japan). Signals on the immunoblot were quantified using a
141 computer program (NIH Image V1.56). Vimentin expression was used as loading control.
142
143 *PGE2, measurements.*
144 Cells were grown and harvested as described above. Cells were then incubated for 24 h with
145 vehicle (control) or with Ang II (0.1 µM) for 4 h in the absence or in the presence of different
146 peptides. The culture media was collected and release of PGE2, was determined using the

147 Prostaglandin E2, ELISA Kit according to the manufacturer's instructions (Cayman Chemical). PGE2
148 concentration was corrected by the protein content of each sample well.
149
150   *Transient transfection assays.*
151   Transient transfections of aortic fibroblasts were performed with LipofectamineTM LTX
152 Reagent (Invitrogen) and the luciferase reporter vector containing 3.9 kb of COX-2 promoter
153 (pGL3-COX-2P) that includes a region from 3074 to 3236 of human COX-2 mRNA cloned in the XbaI
154 site of the pGL3 basic vector, kindly provided by Dr. W. Eberhardt (Klinikum der Johann Wolfgang
155 Goethe-Universität Frankfurt am Main, Germany) [24], with the pCMV-βgal (Clontech, Japan) as
156 internal control vector. Cells were seeded on twelve-well plates (80.000 cells/well) and transient
157 transfection assays were performed with 1 µg plasmid/well, 0.25 µg/well of pCMV-β-gal, 2 µL of
158 LipofectamineTM LTX Reagent and 1 µL Plus reagent following manufacturer's instructions. The
159 complexes DNA/liposome were added to the cells for 18 h. Then cells were incubated overnight in
160 serum free medium and finally stimulated with angiotensin II for 24 hours. Luciferase activity was
161 measured in cell lysates using the Luciferase assay kit (BioThema AB, Handen, Sweden) and a
162 luminometer (Orion II, Berthold detection systems) according to the manufacturer's instructions.
163 Results were normalized by β-galactosidase activity using the Enzyme Assay System (BioThema
164 AB).
165
166   *Statistical analysis.*
167   Data are presented as mean ± standard error (±SE). n indicates the number of experiments.
168 Statistical analysis was done by Student's t test or by one or two-way ANOVA followed by a
169 Bonferroni test. Values were considered to be significant when $P < 0.05$.
170

171 **3. Results and Discussion**

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

180   All peptides studied were ineffective in COX-2 expression in the absence of Ang II. After 4h of
181 incubation with Ang II, COX-2 expression was reduced by several peptides, but only significantly by
182 three sequences (VPP, RDILNQ and YRRGGLEPINF), and the most efficient reduction of COX-2
183 expression was in response to YRRGGLEPINF (Figure 1). The results of our study provide first
184 evidence for the vascular anti-inflammatory activity of egg-derived peptides.
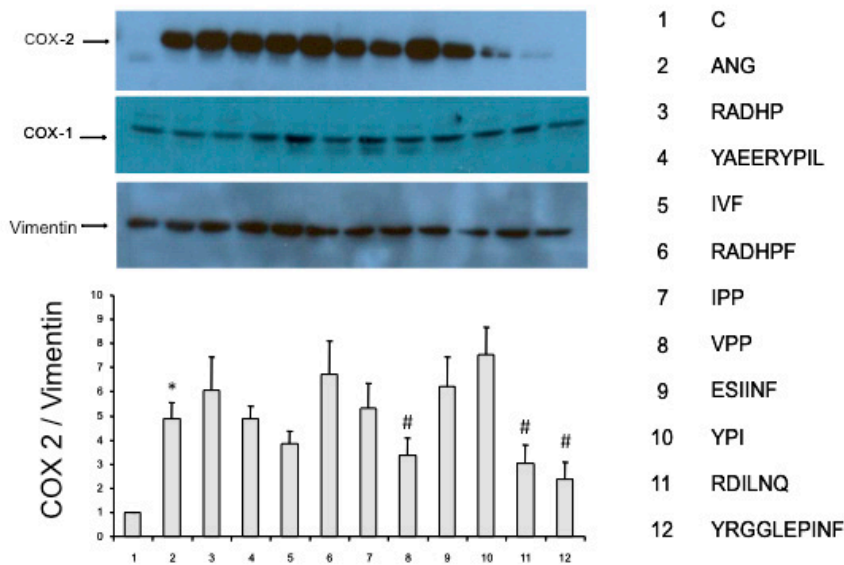185

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Figure 1. Western Blot analysis of ciclooxygenase 2 (COX-2) expression in fibroblast 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.

202

203 To determine whether the effect of these peptides in Ang II-induced COX-2 expression was due
204 to changes in transcription rate, COX-2 transcriptional activity was measured in fibroblasts
205 transiently transfected with a reporter plasmid containing 3900 bp of the human COX-2 promoter
206 fused to luciferase cDNA. Ang II significantly increased the transcriptional activity of the COX-2
207 promoter while the pre-incubation with VPP, RDILNQ or YRGGLEPINF one hour prior to the
208 addition of stimuli, reduced significantly and differentially the luciferase activity. In Ang
209 II-triggered cells, the luciferase activity was nearly reduced to basal levels after incubation with
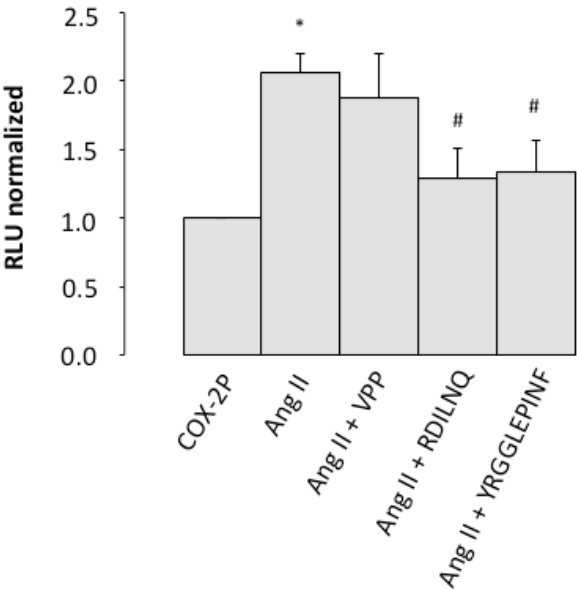210 RDILNQ or YRGGLEPINF but not in the presence of VPP (Figure 2).

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225 **Figure 2.** Luciferase activity (RLU) measured from the transcriptional activation of Ciclooxygenase-2 promoter
226 (pGL3-COX-2P) in aortic fibroblast from Sprague-Dawley rats, by angiotensin II (Ang II) and different food
227 derived peptides (VPP, RDILNQ and YRGGLEPINF). Results were normalized by b-galactosidase activity.
228 Values are means ± SE (n = 4)* P<0.05 Angiotensin II vs control, #P<0.05 Food derived peptides vs Angiotensin
229 II.

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

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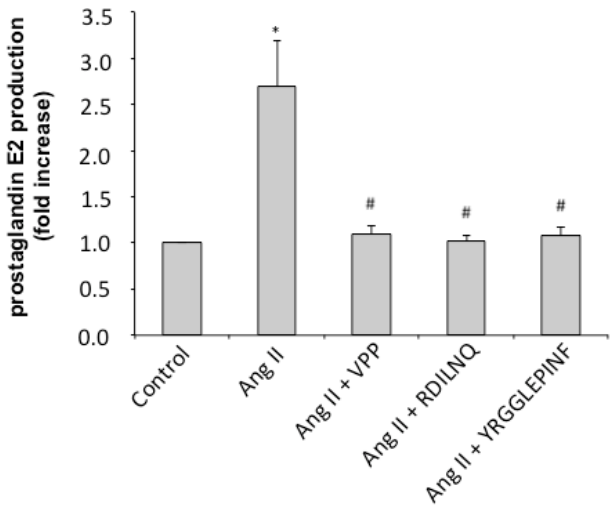248 significantly reduced by the peptides sequences VPP, RDILNQ and YRGGLEPINF (Figure 3).

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

266

267 　　PGE2 production has been related to cardiovascular diseases and this molecule is considered
268 one of the most atherogenic prostanoids, mediating progression of atherogenesis through several

269 mechanisms, such as induction of other pro-inflammatory mediators and adhesion molecules,
270 facilitating migration of macrophages and other immune cells [7].

271 **4. Conclusions**

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

279

280 **Author contributions**

281 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.
282 Analysis and interpretation of data: M.G.-R., M.G., M.S., M.M. Drafting the article: M.G.-R., M.M. Revising the
283 article and approval of the final version to be submitted: M.G.-R., M.G., M.S., M.M.

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288 **Conflicts of interest**

289 The authors declare no conflict of interest.
290

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