Angiotensin-(1–7) reduces proteinuria and diminishes structural damage in renal tissue of stroke-prone spontaneously hypertensive rats

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Submitted 17 May 2010; accepted in final form 15 October 2010

Giani JF, Muñoz MC, Pons RA, Cao G, Toblli JE, Turyn D, Dominici FP. Angiotensin-(1–7) reduces proteinuria and diminishes structural damage in renal tissue of stroke-prone spontaneously hypertensive rats. Am J Physiol Renal Physiol 300: F272–F282, 2011. First published October 20, 2010; doi:10.1152/ajprenal.00278.2010.—Angiotensin (ANG)-(1–7) constitutes an important functional end-product of the renin-angiotensin-aldosterone system that acts to balance the physiological actions of ANG II. In the kidney, ANG-(1–7) exerts beneficial effects by inhibiting growth-promoting pathways and reducing proteinuria. We examined whether a 2-wk treatment with a daily dose of ANG-(1–7) (0.6 mg·kg−1·day−1) exerts renoprotective effects in salt-loaded stroke-prone spontaneously hypertensive rats (SHRSP). Body weight, glycemia, triglyceridemia, cholesterolemia, as well as plasma levels of Na+ and K+ were determined both at the beginning and at the end of the treatment. Also, the weekly evolution of arterial blood pressure, proteinuria, and creatinine clearance was evaluated. Renal fibrosis was determined by Masson’s trichrome staining. Interleukin (IL)-6, tumor necrosis factor (TNF)-α, and nuclear factor-κB (NF-κB) levels were determined by immunohistochemistry and confirmed by Western blotting analysis. The levels of glomerular nephrin were assessed by immunofluorescence. Chronic administration of ANG-(1–7) normalized arterial pressure, reduced glycemia and triglyceridemia, improved proteinuria, and ameliorated structural alterations in the kidney of SHRSP as shown by a restoration of glomerular nephrin levels as detected by immunofluorescence. These results were accompanied with a decrease in both the immunostaining and abundance of IL-6, TNF-α, and NF-κB. In this context, the current study provides strong evidence for a protective role of ANG-(1–7) in the kidney.

THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM (RAAS) is typically visualized as a synchronized hormonal cascade in the control of cardiovascular, renal, and adrenal functions, mainly through the functions of angiotensin (ANG) II, a vasoactive peptide that regulates cell growth, inflammation, and fibrosis through its AT1 receptor. Emerging evidence indicates that ANG II is not the only active peptide of the RAAS. In line with this concept, it is worth mentioning that other members of the RAAS, such as ANG III, ANG IV, and ANG-(1–7), act as important mediators of the various actions of this system (17, 43).

ANG-(1–7) is endogenously formed either from ANG I by cleavage of the Pro7-Phe8 linkage by prolyl endopeptidase, thimet oligopeptidase, or neutral endopeptidase 24.11 or from ANG II by removal of the COOH-terminal phenylalanine by carboxypeptidases, with angiotensin-converting enzyme (ACE) 2 as the primary enzyme (19, 47).

ANG-(1–7) is a heptapeptide that constitutes an important functional end-product of the RAAS that acts to balance the physiological actions of ANG II. In general, ANG-(1–7) acts as a vasodilator agent, equilibrating the proliferative effects of ANG II, and elicits complex renal actions in chronic kidney disease (CKD) and arterial hypertension (30). The Mas G protein-coupled receptor has been shown to bind ANG-(1–7) and is involved in many of its biological actions (46–48). ACE inhibitors elevate ANG-(1–7) concentrations by both increasing ANG I, a substrate for ANG-(1–7) generation, and preventing ANG-(1–7) degradation (19). A large number of studies suggest that, at least in part, the beneficial effects of ACE inhibitors and AT1 receptor blockade could be attributed to ANG-(1–7) (19).

The study of ANG-(1–7) pharmacological properties involves several areas of interest including its effects on blood pressure regulation, cardiac as well as vascular remodeling, cancer and diabetic nephropathy among the main targets of research (30).

Compelling evidence supports the hypothesis that inflammatory cytokines play a decisive role in the development of arterial hypertension and CKD (17). Among them, TNF-α, primarily synthesized by monocytes and macrophages, has been shown to participate significantly in the pathophysiology of diverse renal damage (40). Binding of TNF-α to its receptors in immune and endothelial cells activates nuclear factor-κB (NF-κB) that modulates gene expression of many inflammatory genes including cytokines, chemokines, and growth factors (17). In addition, TNF-α increases expression of other inflammatory factors such as MCP-1 and IL-6 that have been implicated in glomerular and tubulointerstitial injury (40). It has been demonstrated that TNF-α levels are increased in renal diseases such as glomerulosclerosis, immune complex glomerulonephritis, and aminoglycoside nephritis (40). Moreover, renal damage can be reduced by blocking TNF-α activity with neutralizing antibodies or receptor antagonists in diverse models (27, 29, 34).

In the kidney, ANG-(1–7) has been reported to exert beneficial effects. In rat proximal tubular cells, ANG-(1–7) inhibits ANG II-stimulated growth-promoting signaling pathways (15). In renal epithelial LLC-PK cells, high glucose-stimulated protein synthesis and phosphorylation of p38 MAPK are also inhibited by ANG-(1–7) (21). In agreement with these in vitro studies, Benter et al. (6) demonstrated that chronic ANG-(1–7) infusion to streptozotocin-induced diabetic rats reduces proteinuria and improves vascular reactivity in isolated artery segments. Moreover, genetic deletion of the ANG-(1–7) recep-
tor Mas leads to glomerular hyperfiltration, inflammation, and proteinuria (43), further supporting a role for ACE2-ANG-(1–7)-receptor Mas axis in the counterbalance of renal fibrosis exerted by ANG II through its receptor AT1 in this organ.

In the present study, we evaluated the renoprotective effects of 2-wk administration of ANG-(1–7) in the stroke-prone spontaneously hypertensive rat (SHRSP) with moderate load of NaCl, a model of severe arterial hypertension associated with nephropathy and insulin resistance (11, 33, 54). We particularly focused on renal effects of ANG-(1–7) treatment at various levels such as inflammation markers (tissue levels of TNF-α, NF-κB, and IL-6), structural modifications (fibrosis and glomerular abundance of nephrin), and functional effects (proteinuria, creatinine clearance, and arterial blood pressure).

**Materials and Methods**

*Animals and experimental design.* All the experiments were approved by the Animal Care Committee of Hospital Alemán and were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Twelve-week-old male SHRSP and control Wistar-Kyoto rats (WKY; Charles River Laboratories, Wilmington, MA) were housed in individual cages at 21 ± 2°C and a 12:12-h light-dark cycle (7 AM-7 PM). All animals received 1.5% NaCl in their drinking water ad libitum for 2 mo. Then, they were divided into four groups: SHRSP-saline group (n = 8), SHRSP with ANG-(1–7) [SHRSP-ANG-(1–7)] group (n = 8), WKY-saline group (n = 8), and WKY with ANG-(1–7) [WKY-ANG-(1–7)] group (n = 8). During 2 wk, all animals were treated as the following schedule: SHRSP-saline and WKY-saline = receiving no treatment, SHRSP-ANG-(1–7) and WKY-ANG-(1–7) = receiving a daily intraperitoneal dose of ANG-(1–7) (0.6 mg/kg; Bachem Americas, Torrance, CA). Both the dose and administration interval of ANG-(1–7) were selected on the basis of previous studies performed in different animal models including spontaneously hypertensive rats and diabetic hypertensive rats (4–6). All animals were allowed to feed on standard rat chow (18–20% protein; Cooperación-Argentina) ad libitum and they continued receiving 1.5% NaCl in their drinking water ad libitum until the end of the experiment. To evaluate the evolution of proteinuria and creatinine clearance in each group, 24-h urine was collected weekly from baseline to the end of the experiment as well as other biochemical determinations.

**Measurements of blood pressure, blood, and renal function parameters.** At baseline and then weekly, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured by a noninvasive pressure device using volume pressure recording, CODA 2 (Kent Scientific, Torrington, CT). Measurements were obtained in conscious rats restrained in a thermal plastic chamber as described (53). After 14-h fasting, rat blood samples were collected from the tail vein in capillary tubes at baseline and from the inferior cava vein after the rats being killed at the end of the experiment. Plasma glucose level was measured by the glucose oxidase method with an Automatic Analyzer (Hitachi, Tokyo, Japan). Serum samples were stored before testing. Aliquots of sera and urine were assayed for creatinine using the enzymatic UV method (Randox Laboratories, Crumlin, Ireland). Serum cholesterol and triglycerides were assessed according to standard methods. Proteinuria as well as serum and urine electrolytes were determined by standard methods and creatinine clearance was calculated according to the standard formula.

**Tissue collection and histologic study.** At the end of the treatment period, both kidneys of each animal were perfused with saline solution through the abdominal aorta until they were free of blood. For histomorphometrical and immunohistochemical studies, decapsulated kidneys were cut longitudinally, fixed in phosphate-buffered 10% formaldehyde (pH 7.2), and embedded in paraffin. Three-micrometer sections were cut and stained with hematoxin-eosin and Masson’s trichrome. All observations in light microscopy were performed using a light microscope Nikon E400 (Nikon Instrument Group, Melville, NY). A piece of the kidney was preserved at −80°C for immunoblotting determinations.

**Immunohistochemistry.** Paraffin-embedded sections were submitted to immunohistochemical assays as reported (52). Briefly, the sections were deparaffinized with xylene, rehydrated through graded series of ethanol to water, and then incubated in blocking solution (PBS plus 1% bovine serum) at room temperature for 1 h. Then, the sections were incubated overnight at 4°C with one of the following primary antibodies: monoclonal antibody against rat TNF-α dilution (1:50; R&D Systems, Minneapolis, MN), IL-6 (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal anti-NF-κB p65 antibody that detects endogenous level of total NF-κB p65 (1:150 dilution; Santa Cruz Biotechnology), or polyclonal antibody anti-ANG II (1:100 dilution; Phoenix Pharmaceuticals, Belmont, CA). All antibodies were diluted with blocking solution. Negative controls consisted of histological sections incubated with PBS rather than the primary antibody. Immunostaining was carried out with an avidin-biotin-peroxidase complex kit and counterstained with hematoxinil (45). Specificity of the ANG II staining was tested by preincubating the anti-ANG II antibody for 30 min at room temperature with a 100-μM solution of either ANG-(1–14), ANG-(1–7), or ANG II (Bachem Americas).

**Immunofluorescence.** Frozen kidney sections (5 μm) were treated with a goat polyclonal antibody anti-rat nephrin (1:50 dilution; Santa Cruz Biotechnology), followed by a donkey anti-goat IgG-FITC (1:100 dilution; Santa Cruz Biotechnology). Negative controls consisted of histological sections incubated with PBS rather than the primary antibody (53). Immunofluorescence was visualized on a Nikon E400 fluorescence microscope equipped with a high-pressure mercury lamp.

**Immunoblotting.** To determine the protein abundance of IL-6, TNF-α, and NF-κB, equal amounts of solubilized proteins (40 μg) were denatured by being boiled in reducing sample buffer, resolved by SDS-PAGE, and subjected to immunoblotting with the same antibodies used for immunohistochemistry (anti-IL-6 antibody, 1.1.000 dilution; anti-TNF-α antibody, 1.3.000 dilution; anti-NF-κB antibody, 1.3.000 dilution) diluted in Tris-buffered saline 0.1% Tween-20 plus 1% BSA. Finally, membrane blots were washed and incubated for 1 h at room temperature with goat anti-rabbit IgG-horseradish peroxi-

**Table 1. Body weight and blood parameters of experimental animals at baseline**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WKY-Saline</th>
<th>WKY-ANG-(1–7)</th>
<th>SHRSP-Saline</th>
<th>SHRSP-ANG-(1–7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>378 ± 10</td>
<td>376 ± 6</td>
<td>293 ± 6*</td>
<td>295 ± 6*</td>
</tr>
<tr>
<td>Glycemia, mg/dl</td>
<td>105 ± 12</td>
<td>98 ± 14</td>
<td>161 ± 19*</td>
<td>164 ± 26*</td>
</tr>
<tr>
<td>Cholesterolemia, mg/dl</td>
<td>55 ± 9</td>
<td>55 ± 12</td>
<td>59 ± 12</td>
<td>58 ± 10</td>
</tr>
<tr>
<td>Triglyceridemia, mg/dl</td>
<td>66 ± 6</td>
<td>59 ± 10</td>
<td>155 ± 15*</td>
<td>152 ± 13*</td>
</tr>
<tr>
<td>[Na+], meq/l</td>
<td>144 ± 5</td>
<td>145 ± 4</td>
<td>146 ± 5</td>
<td>145 ± 5</td>
</tr>
<tr>
<td>[K+], meq/l</td>
<td>5.4 ± 0.4</td>
<td>5.3 ± 0.5</td>
<td>5.2 ± 0.7</td>
<td>5.3 ± 0.6</td>
</tr>
</tbody>
</table>

Data are means ± SD (n = 8 animals in each group). WKY, Wistar-Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats. *P < 0.01 vs. WKY-saline and WKY-ANG-(1–7).
dase (HRP) secondary antibody (1:20,000 dilution) or donkey anti-goat IgG-HRP secondary antibody (1:10,000 dilution; Santa Cruz Biotechnology). Specific bands were visualized on Kodak X-Omat AR film (Kodak, Rochester, NY) using an enhanced chemiluminescence detection system (GE Healthcare Bio-Sciences) and their intensities were quantitated by digital densitometry.

Morphological analysis. All tissue samples were evaluated independently by two investigators as a single-blind study. All measurements were carried out using an image analyzer Image-Pro Plus ver. 4.5 for Microsoft Windows (Media Cybernetics, LP. Silver Spring, MD, USA). Histological evaluation of kidney was assessed on 20 consecutive microscopic fields at 400 magnification, where each field represents 1.13 mm², resulting in a total explored area of 22.6 mm². Data were averaged and the results were expressed as a percentage per millimeter squared according to the following schedule:
1) ANG II, 2) extracellular matrix (ECM) expansion, 3) IL-6, 4) TNF-α, 5) number of NF-κB p65-positive cells per millimeter squared, and 6) percentage of immunofluorescence for nephrin/glomeruli.

Statistical analysis. Values were expressed as means ± SD. All statistical analyses were performed using absolute values and processed through GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA). The assumption test to determine the Gaussian distribution was performed by the Kolmogorov and Smirnov method. For parameters with Gaussian distribution, comparisons among groups were carried out using two-way ANOVA followed by the Bonferroni’s test. A value of $P < 0.05$ was considered significant.

RESULTS

Metabolic characteristics of the experimental animals; effects of ANG-(1–7) treatment. At the beginning of the 14-day treatment with ANG-(1–7), SHRSP-saline and SHRSP-ANG-(1–7) displayed reduced body weight and increased glycemia and triglyceridemia than those observed in the WKY-saline and WKY-ANG-(1–7) groups. However, no differences were observed in cholesterolemia, Na⁺ and K⁺ plasma levels within the different groups of animals studied (Table 1). At the end of the study, body weight remained decreased in SHRSP-saline and SHRSP-ANG-(1–7). Conversely, SHRSP subjected to the ANG-(1–7) chronic treatment showed an important reduction in glucose plasma levels that reached values not statistically different from those obtained in normal WKY groups. Indeed, the SHRSP-ANG-(1–7) group displayed lower triglyceridemia than the SHRSP-saline group; nevertheless, this reduction did
not reach the WKY normal values (Table 2). Total cholesterol, Na⁺ and K⁺ plasma levels remained unaltered in all experimental groups (Table 2).

**ANG-(1–7) reduces blood pressure and ameliorates proteinuria in SHRSP.** Baseline SBP and DBP in the SHRSP-saline and SHRSP-ANG-(1–7) groups were significantly higher than those displayed by the WKY-saline and WKY-ANG-(1–7) groups. Figure 1 shows the weekly evolution of blood pressure in all groups of animals. In the SHRSP-saline group, both SBP and DBP remained stably high during the entire ANG-(1–7) treatment. After 1 wk of treatment, the levels of SBP and DBP in the SHRSP-ANG-(1–7) group were significantly lower (19 and 18%, respectively; \( P < 0.01 \)) than those observed in the SHRSP-saline group (Fig. 1). With the progression of the study, SBP and DBP decreased progressively in the SHRSP-ANG-(1–7) group; however, at the end of the 2-wk treatment with ANG-(1–7), both values remained significantly higher than the corresponding values obtained in animals belonging to the WKY-saline and WKY-ANG-(1–7) groups (Fig. 1).

At the beginning of the experiment, proteinuria in the SHRSP-saline and SHRSP-ANG-(1–7) groups was significantly higher than that displayed by WKY-saline and WKY-ANG-(1–7) groups. Interestingly, proteinuria was significantly reduced in the SHRSP-ANG-(1–7) group after 1-wk treatment with ANG-(1–7) and remained significantly lower than the untreated SHRSP-saline group by the end of the study (Fig. 1).

Along the 2-wk treatment with ANG-(1–7), creatinine clearance was similar in all experimental groups studied (Fig. 1).

**ANG-(1–7) does not change renal ANG II levels in SHRSP.** As shown in Fig. 2, kidneys from the untreated SHRSP-saline group presented a remarkable higher immunostained ANG II area than that displayed by the WKY-saline group (23 ± 2 vs. 7 ± 1%; \( P < 0.05; n = 8 \)). No differences in the immunostaining levels of ANG II were observed after a 2-wk treatment with ANG-(1–7) (Fig. 2).

**Treatment with ANG-(1–7) reduces renal interstitial fibrosis in SHRSP.** As demonstrated by Masson’s trichrome staining, large areas of fibrosis were detected in kidney of SHRSP-saline, which displayed a significantly greater renal collagen deposition than that observed in the WKY-saline group (22 ± 6 vs. 6 ± 2%; respectively; \( P < 0.05; n = 8 \); Fig. 3). The fibrotic changes in the kidney were significantly reduced in SHRSP submitted to a 2-wk treatment with ANG-(1–7) (66%...
less fibrotic staining compared with SHRSP-saline group; \( P < 0.05; n = 8 \); Fig. 3). No fibrotic areas were observed in WKY rats after treatment with ANG-(1–7) (Fig. 3).

**ANG-(1–7) treatment reduces immunostaining and protein levels of IL-6, TNF-\( \alpha \), and NF-\( \kappa B \) in the kidney of SHRSP.** The evaluation of local proinflammatory cytokines production in the kidney by immunostaining showed that both IL-6 and TNF-\( \alpha \) were increased in kidney of SHRSP-saline (\( P < 0.05; n = 8 \)) compared with WKY-saline rats (Figs. 4 and 5, respectively). Notably, the administration of ANG-(1–7) substantially ameliorated the expression of these cytokines (Figs. 4 and 5). ANG-(1–7) treatment did not modify the expression of IL-6 or TNF-\( \alpha \) in the WKY group (Figs. 4 and 5, respectively). To determine whether changes of these cytokines were accompanied with altered expression of NF-\( \kappa B \) in the kidney, the next goal was to study the renal abundance of the p65 subunit of NF-\( \kappa B \) by immunohistochemistry. As illustrated in Fig. 6, SHRSP-saline showed a significant increase in areas with positive p65 subunit (NF-\( \kappa B \)) immunostaining when compared with the rest of the groups (\( P < 0.05; n = 8 \)). After the 2-wk treatment with ANG-(1–7), the SHRSP-ANG-(1–7) group displayed a reduced protein abundance of NF-\( \kappa B \) in the kidney. As observed with IL-6 and TNF-\( \alpha \), no alterations in NF-\( \kappa B \) expression were evidenced in WKY rats submitted to the ANG-(1–7) chronic treatment (Fig. 6).

**Nephrin expression is restored in kidney from SHRSP after treatment with ANG-(1–7).** As detected by immunofluorescence, SHRSP-saline displayed a dramatic decrease in glomerular nephrin expression compared with the WKY-saline group (\( P < 0.05; n = 8 \); Fig. 7). Interestingly, the group of SHRSP treated with ANG-(1–7) showed a noticeable increase in the expression of renal nephrin reaching values that were indistinguishable from those observed in the control untreated WKY group. In accordance with the previously analyzed parameters, ANG-(1–7) treatment did not alter renal nephrin expression in WKY rats (Fig. 7).

Figs. 3. Masson’s trichrome staining of fibrosis areas in kidney of SHRSP subjected to 1.5% NaCl overload: effect of ANG-(1–7) treatment. Glomerular sections showing extracellular matrix proteins deposition in WKY-saline (A), WKY-ANG-(1–7) (B), SHRSP-saline (C), and SHRSP-ANG-(1–7) (D). E: bars show quantification of fibrosis areas in each group. Data are means ± SD. *\( P < 0.05 \) vs. all groups.
DISCUSSION

Renal fibrosis is observed in both human and experimental models of kidney disease and contributes to progressive decrease in glomerular filtration rate and tubular function (14). In the current study, we show a protective role for ANG-(1–7) in SHRSP, a well-characterized model of arterial hypertension and CKD. We demonstrated that a 2-wk treatment with ANG-(1–7) improves the metabolic profile of this animal model by decreasing glycemia and triglyceridemia to normal values. Hyperglycemia leads to the chronic activation of protein kinase C (PKC), a family of enzymes that have been associated with vascular alterations such as increases in permeability, contractility, ECM synthesis, cell growth and apoptosis, angiogenesis, leukocyte adhesion, and cytokine activation. These alterations in vascular cell homeostasis caused by different PKC isoforms are linked to the development of pathologies affecting large and small vessels leading to nephropathy (22). Regarding the normalization of triglyceridemia observed in SHRSP after the ANG-(1–7) treatment, studies showed that elevated levels of lipids accelerate renal disease progression, which is improved by a variety of manipulations that lower circulating lipids or prevent intracellular lipid accumulation (41). In line with these reports, normalization of plasma glucose and triglycerides could play an important role in the renoprotective actions of ANG-(1–7).

Also, ANG-(1–7) ameliorates renal fibrosis and reduces the expression of inflammatory cytokines in kidney of SHRSP. It has been extensively documented that ANG-(1–7) is capable of exerting a protective effect against target-organ damage in hypertensive situations particularly in the cardiovascular system (5, 23, 25, 35, 38). However, ANG-(1–7) has been shown to exert complex actions in the kidney (15) and whether its effects on this organ are beneficial or detrimental is a matter of controversy. A strong evidence for a protective role for ANG-(1–7) in renal fibrosis has recently been provided by the analysis of the renal effects of knocking-out the Mas receptor.
in mice, which display renal dysfunction associated with increased interstitial fibrosis and upregulation of the tumor growth factor-β mRNA (43). Moreover, ANG-(1–7) treatment has been shown to reduce proteinuria in SHRSP treated with the nitric oxide synthesis inhibitor Nω-nitro-L-arginine methyl ester (4). In a recent study, Zhang et al. (57) showed that ANG-(1–7) is therapeutic in reducing glomeruloesclerosis through counteracting ANG II effects in a rat model of experimental glomerulonephritis.

In contrast, chronic infusion of ANG-(1–7) into rats with streptozotocin-induced diabetes was reported to accelerate renal injury (50), although these results have been recently contradicted (51). In addition, ANG-(1–7) infusion had no protective effect against renal injury in a rat model of nephritic syndrome induced by administration of adriamycin, a commonly used antibiotic that damages renewal systems of highly proliferative cells and also has nephrotoxic actions in experimental animals (50). While differences in dose, experimental design, and physiopathological conditions of experimental animals could contribute to these discrepancies, further studies with different doses of ANG-(1–7) given for a longer term are needed. Moreover, additional evidence against a renoprotective role for the ANG-(1–7)-Mas receptor axis has been recently published. Esteban et al. (18) showed that renal deficiency for Mas receptor diminished renal damage in the models of renal insufficiency of unilateral ureteral obstruction and ischemia-reperfusion injury. Remarkably, systemic infusion of ANG-(1–7) for 5 days into healthy wild-type C57Bl/6 mice induced inflammatory cell infiltration within the kidney. This study indicates that the dose of ANG-(1–7) used might promote renal inflammation under nonpathological circumstances, where there is no exacerbation of the ANG II-AT1 receptor axis (18). However, it is worth mentioning that results reported by Esteban et al. in healthy mice have been obtained after infusion of a very large dose of ANG-(1–7) (1,800 µg·kg⁻¹·day⁻¹), which is approximately 3 to almost 10 times larger than the doses used in previous studies showing beneficial effects of the heptapeptide in various tissues including kidney, heart, and liver. Commonly, doses of ANG-(1–7) used in experimental therapeutic approaches ranged between 144 to 672 µg·kg⁻¹·day⁻¹.

**Fig. 5.** Immunohistochemistry of tumor necrosis factor (TNF)-α in kidney of SHRSP subjected to 1.5% NaCl overload: effect of ANG-(1–7) treatment. Glomerular sections showing TNF-α-positive immunostaining in WKY-saline (A), WKY-ANG-(1–7) (B), SHRSP-saline (C), and SHRSP-ANG-(1–7) (D). E: bars show quantification of TNF-α abundance in each group. F: IB of TNF-α abundance in kidneys of all groups. Data are means ± SD. *P < 0.05 vs. all groups.
irrespective of the way of administration of the hormone (bolus intraperitoneal or chronic infusion through the use of osmotic mini-pumps) (1, 4–6, 23–25, 31, 36, 37, 51, 57). It is well-known that ANG-(1–7) is capable of interacting with AT1 receptors with low affinity (3). Therefore, the results from the study of Esteban et al., which were obtained in healthy mice, suggest that deleterious effects appear when large amounts of ANG-(1–7) are administered. This could be possible due to the ability of AT1 receptors to bind ANG-(1–7) and thus, mimicking the negative actions of ANG II through this receptor.

Inflammatory cytokines have been shown to play a pivotal role in the pathogenesis of hypertension associated with end-stage renal disease (10). The present study provides strong evidence for an anti-inflammatory effect of ANG-(1–7) in the kidney. These results suggest that ANG-(1–7) may exert such a role by inducing a normalization of the levels of proinflammatory cytokines (TNF-α and IL-6) together with a reduction of the transcription factor NF-κB abundance. In support of our current observation, it was demonstrated that ANG-(1–7) induced an attenuation of the NF-κB pathway together with a downregulation of the expression of several proinflammatory cytokine genes, including IL-6, in heart of diabetic hypertensive rats (1).

Protein excretion is an important marker for the progression to end-stage renal disease (42). In the current study, proteinuria was associated to a local increase of inflammation markers such as TNF-α, IL-6, and NF-κB. Interestingly, treatment with ANG-(1–7) totally normalized the levels of proinflammatory cytokines. Furthermore, it is worth mentioning that ANG-(1–7)-treated SHRSP showed a sensible reduction in proteinuria, although not reaching control values. A possible explanation for these findings could be the fact that other cytokines not measured in the current study might be involved in the development of proteinuria. Another possibility could be that the 2-wk treatment with ANG-(1–7) could have been insufficient (mostly in terms of time) to obtain a total control in proteinuria.
The podocyte has emerged as a critical cell type in regulating disease progression of prevalent causes of kidney disease such as diabetes and glomerulosclerosis (14, 26, 49). Podocytes are recognized to play a key role in disease states associated with proteinuria (49). One of the major proteins of the podocyte is nephrin, which functions both as a structural and as signaling molecule in this specialized cell (7). A decreased expression of nephrin correlates with loss of glomerular integrity (7). Thus, the absence of expression of nephrin in the mouse leads to severe podocyte abnormalities and massive proteinuria already in utero (44). Besides its ability to induce hypertension and proteinuria, ANG II decreases mRNA and protein expression of nephrin that plays a key role in the pathogenesis of ANG II-induced podocyte apoptosis (28, 32). However, the mechanism for the induction of the expression of nephrin after infusion of ANG-(1–7) shown in our current study appears to have been proceeded independently of ANG II renal levels, as they remained unchanged after the 2-wk treatment with the heptapeptide. An important factor to be considered as determinant of nephrin expression would be the reduction of blood pressure induced by ANG-(1–7) since recent reports showed that prevention of hypertension precludes the loss of nephrin observed in diabetic states (2). Such a positive modulation of nephrin expression and mitigation of the alteration of the filtration barrier as the one observed in the current study were also demonstrated after treatment with statins (rosuvastatin) (55), ACE inhibitors (8), combined therapy with ACE inhibitors and ET-1 receptor antagonists (20), and also, after treatment of diabetic rats with the PPAR-γ agonist rosiglitazone (39). Still, it is important to point out that in our current study, we show for the first time that the beneficial effects of treatment with ANG-(1–7) in the kidney involve structural aspects as observed by its capacity to induce a positive regulation of nephrin levels in the glomerulus.
We hypothesize that ANG-(1–7) exerts its beneficial changes in the kidney through at least one of the following mechanisms or perhaps a combination of them: 1) a potential inhibitory effect on the signaling cascades leading to the synthesis/production of inflammatory cytokines, 2) improvement of altered metabolic parameters (hyperglycemia and hypertriglyceridemia) that could have led to amelioration of important signaling pathways in the kidney, 3) interference with the ANG II-AT1 receptor signaling system through a mechanism that could involve downregulation of AT1 receptors in the kidney. Such a negative modulation has been shown in vitro both in smooth muscle cells and in kidney slices incubated with increasing concentrations of ANG-(1–7) (12, 13), and finally 4) induction of a reduction in arterial blood pressure, since ANG-(1–7) contributes to the cardio renal control of blood pressure via actions that oppose the activity of ANG II within the heart, kidney, and the blood vessels (9, 19, 46). In addition, the recently discovered ACE2-ANG-(1–7)-Mas receptor axis in the brain seems to act as a neuromodulator in the tonic and reflex control of arterial pressure in many important cardiovascular-related sites in the forebrain, medulla, and hypothalamus, although further research is warranted to clarify the possible physiological role of brain ACE2 and ANG-(1–7) in the modulation of blood pressure and heart rate (16). On the other hand, recent data from acute studies showed that a supraphysiological acute infusion of the ANG-(1–7) did not reduce blood pressure in mice acutely treated with ANG II (56). Indeed, Grobe et al. (24) showed that long-term infusion of ANG-(1–7) significantly decreased cardiac fibrosis and prevented cardiac remodeling in rats chronically infused with ANG II without significant effects on blood pressure. Notwithstanding these observations, in the SHRSP model, the hemodynamic change obtained after ANG-(1–7) treatment (≈50 mmHg) would be undoubtedly favorable leading to improvement in kidney function and reduced inflammation.

In summary, we showed that chronic administration of ANG-(1–7) normalizes SBP and DBP, reduces circulating glucose and triglyceride levels, improves proteinuria, and ameliorates structural (fibrosis and nephron loss) alterations in the kidney of SHRSP. These effects were associated with a noticeable capability of ANG-(1–7) in reducing renal fibrosis and decreasing TNF-α, IL-6, and NF-κB content in this tissue. This beneficial effect proceeded independently of local ANG II levels, since they were increased in the SHRSP both before and after ANG-(1–7) treatment. In this context, the current study provides strong evidence for a protective role of ANG-(1–7) in the kidney.

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