Angiotensin-(1–7) attenuates diabetic nephropathy in Zucker diabetic fatty rats

Jorge F. Giani, Valeria Burghi, Luciana C. Veiras, Analía Tomat, Marina C. Muñoz, Gabriel Cao, Daniel Turyn, Jorge E. Toblli, and Fernando P. Dominici

1Facultad de Farmacia y Bioquímica, Instituto de Química y Fisicoquímica Biológicas, Universidad de Buenos Aires, Buenos Aires, Argentina; 2Facultad de Farmacia y Bioquímica, Cátedra de Fisiología, Universidad de Buenos Aires, Buenos Aires, Argentina; and 3Laboratory of Experimental Medicine, Hospital Alemán, Buenos Aires, Argentina

Submitted 3 February 2012; accepted in final form 2 April 2012

Giani JF, Burghi V, Veiras LC, Tomat A, Muñoz MC, Cao G, Turyn D, Toblli JE, Dominici FP. Angiotensin-(1–7) attenuates diabetic nephropathy in Zucker diabetic fatty rats. Am J Physiol Renal Physiol 302: F1606–F1615, 2012. First published April 4, 2012; doi:10.1152/ajprenal.00063.2012.—Angiotensin (ANG)-(1–7) is known to attenuate diabetic nephropathy; however, its role in the modulation of renal inflammation and oxidative stress in type 2 diabetes is poorly understood. Thus in the present study we evaluated the renal effects of a chronic ANG-(1–7) treatment in Zucker diabetic fatty rats (ZDF), an animal model of type 2 diabetes and nephropathy. Sixteen-week-old male ZDF and their respective controls [lean Zucker rats (LZR)] were used for this study. The protocol involved three groups: 1) LZR + saline, 2) ZDF + saline, and 3) ZDF + ANG-(1–7). For 2 wk, animals were implanted with subcutaneous osmotic pumps that delivered either saline or ANG-(1–7) (100 ng·kg⁻¹·min⁻¹) (n = 4). Renal fibrosis and tissue parameters of oxidative stress were determined. Also, renal levels of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), ED-1, hypoxia-inducible factor-1α (HIF-1α), and neutrophil gelatinase-associated lipocalin (NGAL) were determined by immunohistochemistry and immunoblots. ANG-(1–7) induced a reduction in triglycerides, proteinuria, and systolic blood pressure (SBP) together with a restoration of creatinine clearance in ZDF. Additionally, ANG-(1–7) reduced renal fibrosis, decreased thiobarbituric acid-reactive substances, and restored the activity of both renal superoxide dismutase and catalase in ZDF (41). This attenuation of renal oxidative stress proceeded with decreased renal immunostaining of IL-6, TNF-α, ED-1, HIF-1α, and NGAL to values similar to those displayed by LZR. Angiotensin-converting enzyme type 2 (ACE2) and ANG II levels remained unchanged after treatment with ANG-(1–7). Chronic ANG-(1–7) treatment exerts a renoprotective effect in ZDF associated with a reduction of SBP, oxidative stress, and inflammatory markers. Thus ANG-(1–7) emerges as a novel target for treatment of diabetic nephropathy.

Kidney; neutrophil gelatinase-associated lipocalin; renin-angiotensin system; oxidative stress

DIABETIC NEPHROPATHY is a major microvascular complication of diabetes, representing the leading cause of end-stage renal disease, and a main cause of morbidity and mortality in both type 1 and type 2 diabetic patients (22). The pathophysiological changes in diabetic nephropathy include progressive increase in urinary albumin excretion and a decline in glomerular filtration rate. These renal functional changes are associated with cellular and extracellular derangements in both glomerular and tubulointerstitial compartments (22, 28). Overall, diabetic microvascular complications are caused by prolonged exposure to high glucose levels that lead to mitochondrial overproduction of reactive oxygen species (ROS) (20). The production of ROS is increased in both animal models of diabetes and diabetic human subjects and may play an important role in functional and structural alterations of the kidney (9). Many of the redox-sensitive cytokines [such as interleukin-6 (IL-6)] and chemokines [such as monocyte chemoattractant protein 1 (MCP-1)] are proinflammatory and may play a critical role in the initiation and progression of atherosclerosis and diabetic renal injury (23, 51). In addition, ROS, whether mitochondrial or cell membrane derived, may also be responsible for the activation of classical components of the renin-angiotensin system (RAS), contributing further to the compromise of renal functions (25).

The RAS is classically conceived as a coordinated hormonal cascade in the control of cardiovascular, renal, and adrenal functions, mainly through the actions of angiotensin (ANG) II (39). This octapeptidic hormone is generated by renin and angiotensin-converting enzyme (ACE) not only in the circulation but also produced locally in numerous organs such as kidney, blood vessels, and heart, forming the so-called local RAS (2). Advances in the field led to the recognition of other active components of the RAS metabolism, such as ANG III, ANG IV, and ANG-(1–7) (18, 43), ACE2, which forms ANG-(1–7) directly from ANG II and indirectly from ANG I (16), and the ANG-(1–7)-specific G protein-coupled receptor Mas (44). ANG-(1–7) is a heptapeptidic hormone that constitutes an important functional end product of the RAS that acts to balance the physiological actions of ANG II. Although the renal role of this heptapeptidic hormone remains somewhat controversial (24), it has been reported that ANG-(1–7) infusion produces a marked natriuresis in the kidney of normotensive rats and dogs (14) and causes afferent arteriolar vasodilation (41). Furthermore, treatment either with an ANG-(1–7) monoclonal antibody or with the selective ANG-(1–7) receptor antagonist 7D-Ala-ANG-(1–7) (A-779) elicited a dose-dependent rise in blood pressure and reversed to a significant degree the blood pressure-lowering effects of ACE inhibitors in hypertensive rats (36).

Interestingly, in diabetic hypertensive rats, ANG-(1–7) treatment exerts a renoprotective effect that correlates with a reduction in NADPH oxidase activity (6). In addition, ANG-(1–7) has been shown to attenuate ANG II-stimulated NADPH oxidase-mediated glomerular injury in type 2 diabetic KK-A°/Tg mice (34). Recently, we have shown that chronic administration of ANG-(1–7) improves proteinuria and ameliorates structural alterations (fibrosis and nephritis loss) in the kidney of spontaneously hypertensive stroke-prone rats independently of local ANG II modifications (41). The present study was designed to test the hypothesis that a 2-wk treatment with ANG-(1–7) can exert renoprotective effects in Zucker diabetic fatty rats (ZDF), an animal model of...
diabetes mellitus type 2 that displays a progressive development of nephropathy (13, 26, 30). We focused on the effects of ANG-(1–7) treatment at various levels including metabolic parameters, and expressed as micrograms of NGAL per milligram of urinary creatinine.

### MATERIALS AND METHODS

**Animals and experimental design.** Sixteen-week-old male ZDF and control lean Zucker rats (LZR) (Charles River Laboratories, Wilmington, MA) were housed individually in a controlled environment with a photoperiod of 12 h light-12 h dark (lights on from 0700 to 1900) and a temperature of 20 ± 2°C. Housing, handling, and experimental procedures followed the recommendations in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Pub. No. 85-23, revised 1996) and were approved by the Laboratory Animal Use and Care Committee of the School of Pharmacy and Biochemistry of the University of Buenos Aires. After an acclimatization period of 7 days, rats were randomly divided into three groups: LZR receiving no treatment (LZR + saline; n = 4), ZDF receiving no treatment (ZDF + saline; n = 4), and ZDF treated with ANG-(1–7) (ZDF + ANG-(1–7); n = 4). Over 14 days, both saline and ANG-(1–7) (100 ng kg⁻¹ min⁻¹) were delivered by subcutaneous osmotic pumps (model 2002, Alzet). All animals were allowed standard rat chow (18–20% protein, Cooperación-Argentina) ad libitum and tap water.

**Measurements of blood pressure, renal function, and blood parameters.** At baseline and at the end of the 2-wk treatment, systolic blood pressure (SBP) was measured by a noninvasive pressure device using a Grass polygraph (model 79H, Grass Instrument, Quincy, MA). Measurements were obtained in conscious rats restrained in a thermal plastic chamber as described previously (4). After 6 h fasting, rat blood samples were collected from the tail vein in capillary tubes at room temperature for 1 h. Sections were then incubated overnight at 4°C with one of the following primary antibodies: goat polyclonal anti-IL-6 (dilution 1:50; R&D Systems, Minneapolis, MN), mouse monoclonal antibody anti-ED-1 (dilution 1:100; Serotec, Oxford, UK), mouse monoclonal antibody anti-HIF-1α (dilution 1:100; Novus Biological, Littleton, CO), rabbit polyclonal antibody anti-nitrotyrosine (dilution 1:100; Millipore, Billerica, MA), or polyclonal antibody anti-ANG II (1:100 dilution; Phoenix Pharmaceuticals, Belmont, CA). All antibodies were diluted with blocking solution (PBS + 1% bovine serum) at room temperature for 1 h. Sections were then incubated overnight at 4°C with one of the following primary antibodies: goat polyclonal anti-IL-6 (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal antibody against rat TNF-α (dilution 1:50; R&D Systems, Minneapolis, MN), mouse monoclonal antibody anti-ED1 (dilution 1:100; Serotec, Oxford, UK), mouse monoclonal antibody anti-HIF-1α (dilution 1:100; Novus Biological, Littleton, CO), rabbit polyclonal antibody anti-nitrotyrosine (dilution 1:100; Millipore, Billerica, MA), or polyclonal antibody anti-ANG II (1:100 dilution; Phoenix Pharmaceuticals, Belmont, CA). All antibodies were diluted with blocking solution. Immunostaining was carried out with an avidin-biotin-peroxidase complex kit and counterstaining with hematoxylin (3).

**Tissue collection and histological study.** At the end of the experimental period, animals were anesthetized by an intraperitoneal administration of ketamine-xylazine (50 mg/kg and 1 mg/kg, respectively). As soon as anesthesia was ensured by the loss of pedal and corneal reflexes, both kidneys of each animal were perfused with saline solution through the abdominal aorta until they were free of blood. For histological 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, stained with Sirius red, and illuminated with a polarized light. Histological observations in light microscopy were performed with a Nikon E400 light microscope (Nikon Instrument Group, Melville, NY). All tissue samples were evaluated independently by two investigators without prior knowledge of the group to which the rat belonged. Measurements were carried out with Image-Pro Plus image analyzer version 4.5 for Windows (Media Cybernetics, Silver Spring, MD). A piece of the kidney was preserved at −80°C for immunofluorescence, oxidative stress evaluation, and immunoblotting determinations.

**Immunohistochemistry.** Paraffin-embedded sections were subjected to immunohistochemical assays as reported previously (33). Briefly, the sections were deparaffinized with xylene, rehydrated through graded series of ethanol to water, and then incubated in blocking solution (PBS + 1% bovine serum) at room temperature for 1 h. Sections were then incubated overnight at 4°C with one of the following primary antibodies: goat polyclonal anti-IL-6 (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal antibody against rat TNF-α (dilution 1:50; R&D Systems, Minneapolis, MN), mouse monoclonal antibody anti-ED1 (dilution 1:100; Serotec, Oxford, UK), mouse monoclonal antibody anti-HIF-1α (dilution 1:100; Novus Biological, Littleton, CO), rabbit polyclonal antibody anti-nitrotyrosine (dilution 1:100; Millipore, Billerica, MA), or polyclonal antibody anti-ANG II (1:100 dilution; Phoenix Pharmaceuticals, Belmont, CA). All antibodies were diluted with blocking solution. Immunostaining was carried out with an avidin-biotin-peroxidase complex kit and counterstaining with hematoxylin (3).

**Immunoblotting.** To determine the protein abundance of IL-6, TNF-α, ED-1, HIF-1α, and NGAL, equal amounts of solubilized proteins (40 µg) were denaturated 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-ED-1 antibody, 1:1,000 dilution; anti-HIF-1α, 1:1,000 dilution; anti-NGAL, 1:1,000 dilution). Renal ACE2 protein levels were determined by immunoblotting using an anti-ACE2 antibody acquired from Santa Cruz Biotechnology (cat. # sc-18148). The membranes were incubated with primary antibody and then with secondary antibody conjugated to horseradish peroxidase. Blots were developed with an enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Buckinghamshire, UK). The intensity of the bands was quantified using the Quantity One software (Bio-Rad, Hercules, CA). The relative intensity of each band was normalized to the total protein content of the samples, and the fold change was calculated.

**Table 1. Systolic blood pressure and biochemical parameters of experimental animals**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LZR + Saline</th>
<th>ZDF + Saline</th>
<th>ZDF + ANG-(1–7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>293 ± 20</td>
<td>390 ± 35*</td>
<td>411 ± 37*</td>
</tr>
<tr>
<td>Glycemia, mmol/l</td>
<td>5.8 ± 0.5</td>
<td>16.9 ± 2.2*</td>
<td>16.4 ± 1.5*</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.9 ± 0.2</td>
<td>36.7 ± 2.0*</td>
<td>39.2 ± 2.2*</td>
</tr>
<tr>
<td>Insulin, nmol/l</td>
<td>0.08 ± 0.05</td>
<td>6.2 ± 1.4*</td>
<td>5.7 ± 1.2*</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>3.2 ± 0.1</td>
<td>2.7 ± 0.2*</td>
<td>2.8 ± 0.2*</td>
</tr>
<tr>
<td>Proteinuria, mg/day</td>
<td>3.5 ± 0.8</td>
<td>52 ± 8*</td>
<td>56 ± 10*</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>117 ± 4</td>
<td>130 ± 6*</td>
<td>129 ± 5*</td>
</tr>
<tr>
<td><strong>End of treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>312 ± 15</td>
<td>429 ± 39*</td>
<td>446 ± 44*</td>
</tr>
<tr>
<td>Glycemia, mmol/l</td>
<td>6.0 ± 0.4</td>
<td>19.0 ± 1.7*</td>
<td>17.6 ± 2.2*</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.9 ± 0.5</td>
<td>38.5 ± 3.7*</td>
<td>26.7 ± 2.3*</td>
</tr>
<tr>
<td>Insulin, nmol/l</td>
<td>1.0 ± 0.2</td>
<td>4.0 ± 0.9*</td>
<td>3.3 ± 0.7*</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>3.2 ± 0.1</td>
<td>2.5 ± 0.2*</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>Proteinuria, mg/day</td>
<td>4.9 ± 0.9</td>
<td>94 ± 18*</td>
<td>67 ± 8*</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>114 ± 3</td>
<td>147 ± 5*</td>
<td>129 ± 2*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD. ANG-(1–7); ANGiotensin-(1–7); LZR, lean Zucker rats; SBP, systolic blood pressure; ZDF, Zucker diabetic fatty rats. *P < 0.01 vs. LZR + saline; †P < 0.01 vs. other groups.
from Santa Cruz Biotechnology (1:1,000 dilution). All antibodies were diluted in Tris-buffered saline-0.1% Tween 20 plus 1% BSA. Membranes were then blocked by incubation for 2 h with a blocking buffer composed of Tris-buffered saline-0.1% Tween 20 [10 mmol/l Tris·HCl (pH 7.6), 150 mmol/l NaCl, and 0.02% Tween 20 containing 3% BSA] and were finally incubated for 1 h with the appropriate secondary horseradish peroxidase-coupled antibodies (1:20,000 dilution). Equal protein loading was confirmed by reprobing membranes with anti-β-actin antibody (dilution 1:5,000; Sigma-Aldrich, St. Louis, MO). Specific bands were detected by enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ), and their intensities were quantitated by digital densitometry.

Measurement of oxidative parameters in renal tissue. A fraction of the kidney was homogenized, and lipid oxidative damage was assessed by measuring the extent of TBARS formation and expressed as nanomoles of malondialdehyde per gram of protein (10). Total reduced glutathione content was measured and expressed as nanomoles per milligram of protein (48). SOD activity was assessed by measuring the ability of the homogenate to inhibit autoxidation of epinephrine. Specific activity was expressed as units per milligram of protein. Catalase activity was determined by the conversion of hydrogen peroxide to oxygen and water and was expressed as nanomoles per milligram of protein.

Statistical analysis. Values are expressed as means ± SD. All statistical analyses were performed with 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-Smirnov method. Comparisons among groups were carried out with one-way analysis of variance (ANOVA) followed by Bonferroni’s test. A value of P < 0.05 was considered significant.

RESULTS

Metabolic characteristics of experimental animals. At the beginning of the treatment with ANG-(1–7), ZDF + saline and ZDF + ANG-(1–7) groups displayed increased body weight together with augmented circulating levels of glucose, triglycerides, and insulin compared with LZR + saline rats (Table 1). At the end of the study, body weight, glycemia, and insulinemia remained unaltered between ZDF + saline and ZDF + ANG-(1–7) groups. However, a significantly lower level of triglycerides was evidenced in ZDF submitted to the chronic ANG-(1–7) treatment (Table 1).

Renal functional parameters and systolic blood pressure. Proteinuria and creatinine clearance were determined in experimental animals as indicative of renal function. At baseline, both ZDF + saline and ZDF + ANG-(1–7) groups showed decreased creatinine clearance that correlated with increased proteinuria (Table 1). At the end of the treatment period with ANG-(1–7), creatinine clearance was restored, reaching values not statistically different from those obtained in the LZR + saline group. Additionally, the ZDF + ANG-(1–7) group displayed a significantly better control in urinary protein (Table 1).

Table 2. Oxidative parameters in renal tissue

<table>
<thead>
<tr>
<th></th>
<th>LZR + Saline</th>
<th>ZDF + Saline</th>
<th>ZDF + ANG-(1–7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS, nmol MDA/g protein</td>
<td>161 ± 4</td>
<td>251 ± 16*</td>
<td>158 ± 4</td>
</tr>
<tr>
<td>GSH, nmol/mg protein</td>
<td>35 ± 5</td>
<td>29 ± 3</td>
<td>38 ± 6</td>
</tr>
<tr>
<td>SOD, U/mg protein</td>
<td>6.1 ± 0.9</td>
<td>3.6 ± 0.1*</td>
<td>6.5 ± 1.4</td>
</tr>
<tr>
<td>Catalase, nmol/mg protein</td>
<td>27 ± 8</td>
<td>12 ± 2*</td>
<td>23 ± 2</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD. GSH, reduced glutathione; MDA, malondialdehyde; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances. *P < 0.05 vs. other groups.
As observed by indirect determination of SBP, at the beginning of the study ZDF rats displayed a mild, although significant, increase in SBP. After 14 days of ANG-(1–7) administration, improvements in renal functional parameters were accompanied by a significant reduction in SBP (Table 1).

**Oxidative stress evaluation in kidney homogenates.** To evaluate lipoperoxidation in the kidney of ZDF submitted to a chronic treatment with ANG-(1–7), TBARS were determined. As shown in Table 2, untreated ZDF rats showed a significant increase in lipoperoxidation in the kidney that was restored to control LZR values after treatment with ANG-(1–7). No differences were observed between all analyzed groups regarding renal reduced glutathione total content (Table 2). As observed in Table 2, both catalase and SOD renal enzymatic activities were diminished in the ZDF + saline group, indicating a decreased antioxidant capacity in the kidney of this animal model. After a chronic treatment with ANG-(1–7), a complete normalization of these parameters was observed (Table 2). Nitrotyrosine content was also determined as an additional parameter of renal oxidative stress in ZDF. ZDF treated with saline displayed increased tissue nitrotyrosine levels. As was observed for TBARS, chronic treatment with ANG-(1–7) led to a significant reduction of protein nitration levels in the kidney (Fig. 1).

**Measurement of renal interstitial fibrosis in ZDF.** As observed in Fig. 2, renal sections subjected to Sirius red staining and illuminated with polarized light indicated that the ZDF + saline group presented an increased degree of fibrosis compared with the LZR control group. Interestingly, ZDF chronically treated with ANG-(1–7) showed a significant reduction in the expansion of extracellular matrix (ECM) proteins, reaching values not statistically different from LZR (Fig. 2).

**Determination of inflammatory markers in kidney of ZDF: effects of chronic treatment with ANG-(1–7).** The reduction in renal fibrosis was accompanied by a decreased production of local proinflammatory markers. Accordingly, ZDF + saline rats displayed an increased abundance of IL-6, TNF-α, ED-1, and HIF-1α compared with LZR. As was observed for renal fibrosis, ANG-(1–7) treatment led to a reduction of these inflammatory markers in the kidney (Fig. 3 and Table 3). Immunostaining results were confirmed by immunoblotting analysis of kidney homogenates (Fig. 4).

**Renal and urinary content of NGAL.** The local expression of NGAL, a marker of renal tubular damage, was also examined by immunohistochemical and immunoblotting analysis. As observed in Fig. 5, A and B, nontreated ZDF presented an increased renal expression of NGAL compared with the LZR + saline group. As was observed for other inflammatory markers evaluated, chronic treatment with ANG-(1–7) reduced the expression of NGAL in the kidney of ZDF (Fig. 5A). This result was confirmed by immunoblotting (Fig. 5B). Urinary abundance of this premature inflammatory marker was also evaluated by ELISA. As demonstrated in Fig. 5C, increased local NGAL expression in the ZDF + saline group was associated with the augmented presence of this protein in urine. Treatment with ANG-(1–7) restored urinary levels of NGAL to values not statistically different from control LZR.

**Angiotensin II renal levels in ZDF after treatment with ANG-(1–7).** To determine whether ANG-(1–7)-related beneficial effects were associated with decreased local abundance of ANG II, immunostaining analysis of this hormone was performed in the kidney of all groups of rats. Interestingly, both ZDF + saline and ZDF + ANG-(1–7) groups displayed increased levels of ANG II compared with the LZR + saline group, indicating that renopro-

---

**Fig. 2.** Sirius red staining illuminated with polarized light to evidence fibrosis areas in kidney of ZDF subjected to a chronic treatment with ANG-(1–7). Kidney sections show extracellular matrix protein deposition (arrows) in LZR + saline (A), ZDF + saline (B), and ZDF + ANG-(1–7) (C) groups. D: quantification of fibrosis areas in each group. Data are means ± SD. *P < 0.01 vs. other groups.
Fig. 3. Kidney sections show immunohistochemical staining (arrows) of interleukin-6 (IL-6; A), tumor necrosis factor-α (TNF-α; B), ED-1 (C), and hypoxia-inducible factor-1α (HIF-1α; D) in the kidney of ZDF subjected to chronic treatment with ANG-(1–7).
Table 3. Immunohistochemical analysis of inflammatory markers in kidney at end of experiment

<table>
<thead>
<tr>
<th></th>
<th>LZR + Saline</th>
<th>ZDF + Saline</th>
<th>ZDF + ANG-(1–7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>1.8 ± 0.8</td>
<td>16.2 ± 1.9†</td>
<td>2.9 ± 1.1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.2 ± 0.6</td>
<td>19.8 ± 2.4†</td>
<td>5.6 ± 1.1*</td>
</tr>
<tr>
<td>ED-1</td>
<td>1.1 ± 0.5</td>
<td>8.5 ± 1.2†</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>2.1 ± 0.5</td>
<td>19.7 ± 2.4†</td>
<td>3.0 ± 0.9</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD percentage per area. HIF-1α, hypoxia-induced factor-1α; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α. *P < 0.01 vs. LZR + saline; †P < 0.01 vs. other groups.

Protective effects exerted by ANG-(1–7) were independent of local ANG II levels (Fig. 6).

Protein abundance of ACE2 in kidney of ZDF subjected to chronic treatment with ANG-(1–7).

To gain more insight into the modulation of the local RAS in the kidney of ZDF, ACE2 protein levels were determined by immunoblotting assay. As observed in Fig. 7, no statistical difference was observed in the renal abundance of ACE2 within the different groups evaluated in the study.

DISCUSSION

Diabetic nephropathy is a major microvascular complication of diabetes, and it is characterized by excessive accretion of ECM in the kidney that progresses to glomerulosclerosis, tubulointerstitial fibrosis, and, ultimately, renal failure (28). In this study, we determined that ANG-(1–7) induced a protective role in the kidney of ZDF, an animal model of type 2 diabetes mellitus and progressive nephropathy. This was evidenced by a reduction in proteinuria and a complete restoration of creatinine clearance, together with a discrete, but significant, decrease in SBP. Several works have demonstrated that ANG-(1–7) exerts a protective role against endothelial dysfunction and target-organ damage in diabetes, especially in the cardiovascular and renal systems (5, 6, 15, 34, 46). In addition, a protective role for ANG-(1–7) was evidenced in Mas receptor knockout mice, which display renal dysfunction associated with increased interstitial fibrosis and upregulation of transforming growth factor-β mRNA (40). In line with these reports, in the present study we determined that a chronic subcutaneous perfusion of ANG-(1–7) ameliorated renal fibrosis and reduced the expression of inflammatory cytokines in the kidney of ZDF.

Hyperglycemia has been considered to perform a central role in diabetes-associated vascular injury (28). Interestingly, the present study provides substantial information supporting a protective role of ANG-(1–7) in the kidney of ZDF that proceeded without modifications in glycemia and/or insulinemia. Free fatty acids are elevated in insulin-resistant states and...
may directly contribute to cellular insulin resistance. Exposure to palmitate has been shown to reduce insulin signaling and induce insulin resistance in podocytes, suggesting a potential link between systemic insulin resistance and the development of nephropathy (32). In agreement with this observation, increased lipidemia has been consistently associated with renal damage (27) and pharmacological reduction of serum lipids leads to a reduction of glomerular injury (29). Thus, in part, our present results showing a glycemia-independent mechanism for antioxidative and anti-inflammatory effects of ANG-(1–7) in Zucker diabetic rats could be related to control of triglyceride plasma concentrations.

When considering the mechanisms underlying hyperglycemia-induced diabetic vascular damage, a major role has been ascribed to the increased production of mitochondrially derived ROS (20, 28). Overproduction of ROS is associated with diabetes mellitus and hypertension, and recent studies suggest that oxidative stress contributes to the enhanced renal damage and dysfunction in the diseased kidney (21). In line with these reports, it was demonstrated that damage to the renal cortex induced by a high-fructose diet in obese Zucker rats is related to increased oxidative stress and inflammation (17). Our present results show that ANG-(1–7) treatment reduced TBARS and restored the activity of antioxidant enzymes (i.e., SOD and catalase) in the kidney of ZDF. Thus this amelioration of renal oxidative stress could represent a possible mechanism by which ANG-(1–7) exerts its renal protective effects.

Nitration of protein tyrosine residues has been detected in different pathologies, including diabetes, hypertension, and atherosclerosis, all associated with enhanced oxidative stress (49). In agreement with these observations, our present results show that the amelioration of renal oxidative state induced by ANG-(1–7) was associated with decreased renal nitrotyrosine content in ZDF.

Glomerular or interstitial inflammation is thought to play an important role in the initiation of renal fibrosis and chronic kidney disease. Release of inflammatory cytokines has been shown to play a pivotal role in the pathogenesis of diabetes-associated end-stage renal failure (11). The present results provide strong evidence for an anti-inflammatory effect of ANG-(1–7) in the kidney, as evidenced by its ability to normalize the levels of several proinflammatory cytokines (IL-6,
ED-1, and TNF-α). In support of our present observations, it was demonstrated that ANG-(1–7) was effective in attenuating diabetic-associated cardiac dysfunction, possibly through a mechanism that involved an attenuation of the NF-κB pathway together with a downregulation of the expression of several proinflammatory cytokine genes, including IL-6, in the heart of diabetic hypertensive rats (1). One of the major factors underlying vascular inflammation is the modulation of proinflammatory gene expression via redox-sensitive transcription factors (38). Thus amelioration of oxidative stress exerted by ANG-(1–7) in ZDF could be an additional contributor to the reduced expression of inflammatory markers.

It was shown that renal tubular cells may produce and release NGAL in response to various injuries. NGAL levels predict the future appearance of acute kidney injury after treatments potentially detrimental to the kidney and even the acute worsening of unstable nephropathies (7). Recent evidence also suggests that NGAL may somehow be involved in the pathophysiological process of chronic renal diseases such as polycystic kidney disease and glomerulonephritis (7). Furthermore, NGAL might play an important role in the pathophysiology of renal adaptation to diabetes, probably as a defensive mechanism aiming to mitigate tubular suffering (8). A close relationship was established between inflammatory processes and renal NGAL production (12, 52). In line with these reports, the present findings show that the increased abundance of renal inflammatory markers observed in ZDF is associated with augmented renal abundance and urinary release of NGAL. Interestingly, the beneficial effects of chronic administration of ANG-(1–7) correlate with a decreased expression and urinary content of this renal injury biomarker. Recently, an association between NGAL and HIF-1α was established in the progression of chronic kidney disease (50). Our present results support this observation since restoration of both tissue and urinary levels of NGAL induced by ANG-
(1–7) in kidney of ZDF was accompanied by a critical reduction of HIF-1α renal expression.

The RAS, and particularly its main effector peptide, ANG II, has an important role in the development and progression of diabetic nephropathy (47). Inhibitors of the RAS such as ACE inhibitor (ACEI) or an ANG II receptor blocker (ARB) display renoprotective effects in patients with diabetic nephropathy (47). ANG II and oxidative stress, separate or in combination, can lead to the development of inflammation and fibrosis of the kidney (42). Accordingly, it was demonstrated that ANG II plays an important role in modulating the expression of proinflammatory molecules, such as IL-6, through a mechanism that requires the production of ROS and activation of the redox-regulated transcription factor NF-κB (45). Advances in the field led to the recognition of other active components of RAS metabolism, such as ANG-(1–7) (18, 43), ACE2, which forms ANG-(1–7) directly from ANG II and indirectly from ANG I (16), and the ANG-(1–7) specific G protein-coupled receptor Mas (44). The ACE2/ANG-(1–7)/Mas receptor axis seems to accelerate the development of diabetic nephropathy (44). Advances in this field led to the recognition of other active components of RAS metabolism, such as ANG-(1–7) (18, 43), ACE2, which forms ANG-(1–7) directly from ANG II and indirectly from ANG I (16), and the ANG-(1–7) specific G protein-coupled receptor Mas (44). The ACE2/ANG-(1–7)/Mas receptor axis in general opposes the vascular and proliferative effects of ANG II (43). Indeed, a large number of studies suggest that endogenous levels of ANG-(1–7) may contribute to the renoprotective actions of blockade of ANG II since ACEI and ARB increase renal ACE2 activity and ANG-(1–7) formation (19).

In agreement with these findings, treatment with human recombinant ACE2 ameliorated diabetic nephropathy and decreased blood pressure and NADPH oxidase activity in a mouse model of type 1 diabetes (37). Conversely, loss of ACE2 seems to accelerate the development of diabetic nephropathy in type 1 diabetic mice (53). In the present study it was determined that ACE2 protein levels were similar in LZR and ZDF and that treatment of ZDF with ANG-(1–7) did not modify ACE2 protein levels in the kidney. In line with these observation, Leehey and coworkers found similar glomerular ACE2 levels between LZR and ZDF (31). Furthermore, recently published data showed that female mRen2.Lewis rats are not protected from vascular damage, renal inflammation, and kidney injury in early STZ-induced diabetes despite displaying a marked increase in circulating ACE2 and significantly reduced ACE within the kidney (54). Taken together, our present results suggest that the positive renal effects of ANG-(1–7) appear to mimic those of ACE2 therapy and proceed even in the absence of changes in renal ACE2 protein abundance.

Regarding ANG II tissue levels, immunohistochemical analysis revealed a significant increase of positive staining area for this hormone in the kidney of ZDF that remained unaltered after the chronic treatment with ANG-(1–7). As observed for ACE2, the mechanism behind the renoprotective effect of ANG-(1–7) in ZDF appears to be independent of ANG II renal levels, since they remained unchanged after the 2-wk treatment with the heptapeptide ANG-(1–7).

In conclusion, we have shown that chronic administration of ANG-(1–7) to ZDF reduced circulating triglycerides, proteinuria, and SBP and induced a complete restoration of creatinine clearance. Additionally, we determined that ANG-(1–7) was able to rescue the oxidative stress in this animal model together with a substantial reduction of tissue expression of proinflammatory markers. These beneficial effects proceeded independently of local ACE2 or ANG II levels. Our present results provide strong evidence for a protective role of ANG-(1–7) in diabetic nephropathy.

ACKNOWLEDGMENTS

F. P. Dominici, M. C. Muñoz, J. E. Toblli, A. Tomat, and D. Turyn are researchers at the National Research Council of Argentina (CONICET). J. F. Giani is a research fellow from CONICET. V. Burghi and L. C. Veiras are undergraduate fellows from Universidad de Buenos Aires (UBA).

GRANTS

This work was supported by grants from UBA (B051, B080, and 20020100100207) and CONICET (PIP 114–200801–00374).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


