Angiotensin-(1–7) improves cardiac remodeling and inhibits growth-promoting pathways in the heart of fructose-fed rats

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ANGIOTENSIN (ANG) II CONTRIBUTES to insulin resistance and other components of the cardiometabolic syndrome such as hypertension and dyslipidemia (36). In addition, ANG II plays a critical role in the genesis of cardiac hypertrophy, interstitial fibrosis, and left ventricular dysfunction (34, 37). Although this is a critical issue and focus of intense research, the molecular mechanisms linking insulin resistance to hypertension and cardiovascular disease are not well understood (31).

Besides its ability to induce myocyte hypertrophy, several studies (33) suggest that ANG II promotes fibroblast proliferation due to activation of the ANG II type 1 receptor (AT1R), which results in increased collagen type III expression and accumulation in the heart. The mitogen-activated protein kinases (MAPKs), a family of cytosolic serine/threonine kinases that are involved in the regulation of cell growth and differentiation, have been extensively implicated in promoting the ANG II-induced cell proliferation and cardiac myocyte hypertrophy (26, 48). Among the best-characterized mammalian MAPKs are 1) the 42- and 44-kDa extracellular signal-regulated kinases (ERK2 and ERK1, respectively); 2) the c-Jun amino-terminal kinase 1/2 (JNK1/2); and 3) the p38MAPK (16). The ERK1/2 cascade is activated in cardiac myocytes by virtually all hypertrophic stimuli, including growth factors and ANG II via the AT1R (1, 2, 43). Unlike ERK1/2, JNK1/2 and p38MAPK are weakly activated by growth factors but markedly activated in response to the inflammatory cytokines, TNF-α, ANG II and a variety of cellular stresses, such as ischemia/reperfusion (3, 23, 32, 49).

ANG-(1–7) is a heptapeptide that constitutes an important functional end product of the renin-angiotensin system and is primarily formed from ANG II by ACE2 (7, 40). Through its specific G protein-coupled receptor Mas, ANG-(1–7) exhibits cardioprotective effects (17, 38) and induces responses opposing those of ANG II, including antihypertrophic, antihypertrophic, antibiogenic, and antithrombotic properties (7, 19, 40). ANG-(1–7) was found to reduce the incidence and the duration of reperfusion arrhythmias (8). In addition, this heptapeptide preserves cardiac function, coronary perfusion, and aortic endothelial function in a rat model for heart failure (24). In the DOCA-salt hypertensive rat model, ANG-(1–7) was effective in preventing the cardiac myocardial and perivascular fibrosis (15). Consistent with the antiproliferative effects reported for ANG-(1–7), Grobe et al. (14) demonstrated that ANG-(1–7) prevents ANG II-induced cardiac remodeling in adult rats. Tallant et al. (45) reported that this heptapeptide inhibits protein synthesis in cardiac myocytes through activation of the cardiac specific ANG-(1–7) receptor Mas. This inhibition was associated with reduced activity of ERK1/2. This correlated well with our previous demonstration that in vivo acute administration of ANG-(1–7) prevents the ANG II-induced stimulation of ERK1/2 in rat heart through the Mas receptor (11). Recently, it was demonstrated that Src-homology 2-containing protein-tyrosine phosphatase-1 (SHP-1) and -2 (SHP-2) could be involved in MAPKs inactivation. Gava et al. (9) showed that ANG-(1–7) blocks high glucose-induced p38MAPK...
activation, an effect associated with activation of SHP-1. In line with these reports, studies in human endothelial cells indicate that ANG-(1–7) negatively regulates ANG II signaling by enhancing the SHP-2 activity (39). These findings support a putative physiological function of ANG-(1–7) in cell growth. Accordingly, in the present study, we examined whether chronic treatment with ANG-(1–7) inhibits growth-promoting pathways and reverts cardiac hypertrophy and fibrosis in FFR, an animal model of insulin resistance with mild hypertension, elevated circulating levels of ANG II (18), and left ventricular hypertrophy (21). We provide molecular evidence for ANG-(1–7)-induced improvement in cardiac remodeling and provide new insights into the molecular mechanisms involved in the antihypertrophic effects of ANG-(1–7) in the heart of insulin-resistant rats.

METHODS

Materials. The peptide ANG-(1–7) was synthesized in our laboratory as described previously (10). The reagents and apparatus for SDS-PAGE were obtained from Bio-Rad (Hercules, CA). The apparatus for immunoblotting was purchased from Schleicher & Schuell (Southam, England). The rabbit antibodies to unphosphorylated ERK1/2, anti-p38MAPK, anti-phospho-specific p38MAPK, goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP), and goat anti-mouse IgG-HRP secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit antibody to unphosphorylated JNK1/2 and the anti-phospho-specific ERK1/2 and JNK1/2 antibodies were purchased from Cell Signaling (Beverly, MA). The polyclonal antibody anti-AT1R was acquired from Abcam (Cambridge, MA). The mouse antibodies to SHP-1 and SHP-2 were purchased from BD Transduction Laboratories (San Jose, CA). The mouse monoclonal anti-collagen III antibody was acquired from Biogenex (San Roman, CA). The rabbit antibody anti-ANG II antibody was purchased from Phoenix Pharmaceuticals (Belmont, CA). The rabbit polyclonal anti-Mas receptor was acquired from Novus Biologicals (Littleton, CO). ECL was from Amersham (Piscataway, NJ). ANG II RIA kit was purchased from Biosource (Belmont, CA). The rabbit policlonal anti-ANG II antibody was purchased from Phoenix Pharmaceuticals (Belmont, CA). The rabbit antibody anti-ANG II antibody was purchased from Sigma Chemical (St. Louis, MO).

Animals and treatments. A total of 32 male Sprague-Dawley rats weighing 220–240 g were used for this study. All animals were housed individually in a controlled environment with a photoperiod of 12-h light:12-h dark (lights on from 0600 to 1800 h) and a temperature of 20 ± 2 °C. Housing, handling, and experimental procedures followed the recommendations written in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health [DHEW Publication No. (NIH) 85-23, Revised 1996, Office of Science and Health Reports, DRR/NIH, Bethesda, MD] and were approved by the Laboratory Animal Use and Care Committee of the University of Buenos Aires. Following an acclimatization period of 7 days, rats were randomly divided into two groups, a control group that received regular diet (19% protein, 77% carbohydrate, and 4% fat) and a fructose-fed group that was fed a regular diet together with fructose that was administered as a 10% solution (prepared every 2 days) in drinking water during 6 wk as previously described (25). Control animals were given ordinary tap water to drink throughout the entire experimental period. Animals consumed diets and fluids ad libitum. For the last 2 wk of either feeding period, 8 animals from the control group and 8 from the fructose-fed group were implanted with subcutaneous osmotic pumps (model 2002; Alzet, CA) that delivered ANG-(1–7) (100 ng·kg⁻¹·min⁻¹). Pumps were implanted subcutaneously on the back between the shoulder blades and hips while the animals were anesthetized with a mixture of ketamine and xylazine (50 and 1 mg/kg, respectively). The rest of the animals (8 in each group) underwent a sham surgery that involved the performance of a small incision on the back and one stitch without placement of an osmotic pump.

Determination of blood parameters. All measurements were determined 6 h after food removal. Blood glucose measurements were performed using a hand-held glucometer (Accucheck, Mannheim, Germany). Insulin levels were assessed using a rat insulin ELISA kit (Ultra Sensitive Rat Insulin ELISA Kit; Crystal Chemicals). Blood samples for measurement of ANG II plasma levels were collected in polypropylene tubes containing 10 mM p-hydroxymercury benzoate, 15 mM 1,10-phenanthroline, 1 mM peptatin A, and 7.5% EDTA (50 μL/mL of blood). ANG II plasma levels were measured by an ANG II-RIA kit.

Surgical procedure and heart removal. By the end of the experimental period, animals were anesthetized by the intraperitoneal administration of ketamine/xylazine and submitted to the surgical procedure as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The thorax was opened, and the entire heart was removed and weighted. The heart-to-body weight ratio was determined for each animal individually.

Morphological analysis. A portion of the left ventricle was fixed in phosphate-buffered 10% (vol/vol) formaldehyde (pH 7.2) and embedded in paraffin. For myocardial diameter determination, three-micron sections were obtained from the left ventricular equator and hematoxylin-eosin stained. Measurements (×400 magnification) were assessed on 10 consecutive microscopic fields per section and expressed as myocyte diameter (μm). Four left ventricle sections from each experimental animal were stained.

Tissue homogenization and Western blotting analysis. The heart was homogenized in solution buffer containing 1% Triton together with phosphatase and protease inhibitors as described previously (10, 29). Tissue extracts were centrifuged at 100,000 g for 1 h at 4 °C to eliminate insoluble material, and protein concentration in the supernatants was measured using the Bradford method as described previously (10, 29). To evaluate phosphorylation levels of ERK1/2, JNK1/2, and p38MAPK, equal amounts of solubilized proteins (40 μg) were denatured by boiling in reducing sample buffer, resolved by SDS-PAGE, and transferred (Western blot) to PVDF membranes as previously described (10, 29). Membranes were blocked by incubation for 2 h with TBS containing 0.1% Tween 20 and 3% BSA and subsequently incubated overnight with the corresponding phospho-specific antibodies (1:1,000 dilutions). Protein abundance was detected by reprobing membranes with the corresponding antibodies (1:1,000 dilutions). To evaluate AT1R and Mas receptor protein abundance, equal amount of proteins were subjected to immunoblotting with anti-AT1R or anti-Mas receptor antibodies, respectively, as described above. Equal protein loading in the gels was confirmed by reblotting the same membranes with an anti-β-actin antibody. After being extensively washed, all membranes were incubated with the appropriate secondary HRP-coupled antibodies and processed for enhanced chemiluminescence using the ECL plus Western blotting detection system (Amersham Biosciences, Piscataway, NJ). Bands were quantified using Gel-Pro analyzer 4.0 (Media Cybernetics, Bethesda, MD).

Table 1. Body weight and circulating levels of glucose, insulin, and ANG II in the experimental animals at the end of the study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham</th>
<th>ANG-(1–7)</th>
<th>Control</th>
<th>Fructose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weigh, g</td>
<td>361 ± 7</td>
<td>331 ± 12</td>
<td>344 ± 15</td>
<td>365 ± 9</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>120 ± 6</td>
<td>117 ± 4</td>
<td>121 ± 7</td>
<td>114 ± 4</td>
</tr>
<tr>
<td>Plasma insulin, ng/ml</td>
<td>1.3 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>3.0 ± 0.4*</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>ANG II, pmol/l</td>
<td>94 ± 18</td>
<td>116 ± 19</td>
<td>215 ± 32*</td>
<td>194 ± 20*</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 8 animals in each group). *P < 0.05 vs. all groups by two-way ANOVA.
SHP-1 and SHP-2 activity measurements. These determinations were performed according to a previously described protocol (9). Equal amounts of solubilized heart protein (2 mg) were incubated at 4°C overnight with anti-SHP-1 or anti-SHP-2 antibodies at a final concentration of 4 μg/ml. Immune complexes were collected by incubation with protein G-agarose. The beads were washed and mixed with 100 μl of protein tyrosine phosphatase buffer (25 mM imidazole pH 7.2, 45 mM NaCl, and 1 mM EDTA) containing 30 mM p-nitrophenyl phosphate (pNPP), followed by incubation at 37°C for 30 min. The rate of formation of p-nitrophenol from pNPP was determined by spectrophotometry at 410 nm. For each assay of protein tyrosine phosphatase activity, the absorbance of pNPP hydrolyzed from beads containing a control IgG alone was subtracted from the absorbance of lysate immunoprecipitates. After measurement of protein tyrosine phosphatase activity, the beads were recovered and subjected to SDS-PAGE for immunoblotting to measure the protein abundance of SHP-1 or SHP-2.

Interstitial fibrosis determination. A portion of the left ventricle was fixed in phosphate-buffered 10% (vol/vol) formaldehyde (pH 7.2) and embedded in paraffin. Three-micron sections were stained with Masson’s trichrome to evaluate fibrosis. Measurements (×400 magnification) were assessed on 10 consecutive microscopic fields per section and expressed as a percentage of interstitial fibrosis per millimeter square. Four left ventricle sections from each experimental animal were stained.

![Control – sham](image1)

![Control – ANG-(1-7)](image2)

![Fructose – sham](image3)

![Fructose – ANG-(1-7)](image4)

![E](image5)

Table 2. Systolic blood pressure and parameters of cardiac hypertrophy in the experimental animals at the end of the study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>ANG-(1–7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP, mmHg</td>
<td>115 ± 11</td>
<td>137 ± 10*</td>
</tr>
<tr>
<td>HW/BW, 10⁻³</td>
<td>2.73 ± 0.04</td>
<td>3.1 ± 0.1*</td>
</tr>
<tr>
<td>Myocyte diameter, μm</td>
<td>16.3 ± 0.3</td>
<td>17.5 ± 0.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 8 animals in each group). SBP, systolic blood pressure; BW, body weight; HW, heart weight. *P < 0.05 vs. all groups by two-way ANOVA.

SHP-1 and SHP-2 activity measurements. These determinations were performed according to a previously described protocol (9). Equal amounts of solubilized heart protein (2 mg) were incubated at 4°C overnight with anti-SHP-1 or anti-SHP-2 antibodies at a final concentration of 4 μg/ml. Immune complexes were collected by incubation with protein G-agarose. The beads were washed and mixed with 100 μl of protein tyrosine phosphatase buffer (25 mM imidazole pH 7.2, 45 mM NaCl, and 1 mM EDTA) containing 30 mM p-nitrophenyl phosphate (pNPP), followed by incubation at 37°C for 30 min. The rate of formation of p-nitrophenol from pNPP was determined by spectrophotometry at 410 nm. For each assay of protein tyrosine phosphatase activity, the absorbance of pNPP hydrolyzed from beads containing a control IgG alone was subtracted from the absorbance of lysate immunoprecipitates. After measurement of protein tyrosine phosphatase activity, the beads were recovered and subjected to SDS-PAGE for immunoblotting to measure the protein abundance of SHP-1 or SHP-2.

Interstitial fibrosis determination. A portion of the left ventricle was fixed in phosphate-buffered 10% (vol/vol) formaldehyde (pH 7.2) and embedded in paraffin. Three-micron sections were stained with Masson’s trichrome to evaluate fibrosis. Measurements (×400 magnification) were assessed on 10 consecutive microscopic fields per section and expressed as a percentage of interstitial fibrosis per millimeter square. Four left ventricle sections from each experimental animal were stained.

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Immunohistochemical staining. Paraffin sections were cut in 3-μm widths, deparaffinized, and rehydrated. Endogenous peroxidase activity was blocked by treatment with 0.5% H2O2 in methanol for 30 min. To evaluate collagen type III, ANG II, AT1R, and Mas receptor levels in myocardium, specific antibodies were used at dilutions 1:100. Specificity of the ANG II staining was tested by preincubating the anti-ANG II antibody with rat ANG II. Sections were immunostained with a commercial modified avidin-biotin-peroxidase complex technique and counterstained with hematoxylin. The samples were handled as previously described (17, 27a). Four left ventricle sections from each experimental animal were stained. On each section, 10 consecutive microscopic fields (×400 magnification) were analyzed to evaluate collagen type III, ANG II, AT1R, and Mas receptor immunostaining density in the heart. The staining was expressed as density per millimeter square.

In all cases, two independent observers performed a blinded fashion evaluation, and the mean percentage value was then calculated for each rat. All histological sections were studied in each animal using a light microscope Nikon E400 (Nikon Instrument Group, Melville, NY). Measurements were carried out using an image analyzer Image-Pro Plus 4.5 for Windows (Media Cybernetics).

Systolic blood pressure determination. The rats were trained to the procedure of systolic blood pressure (SBP) measurement at 1,300 twice/wk for 2 wk previous to the final measurement. The mean of 10 consecutive readings was used as the reported value of the SBP for each rat. Indirect SBP was measured at week 4 and week 6 by means of the tail-cuff method using a blood pressure analysis system (model SC1000; Hatteras Instruments, Cary, NC).

Statistical analysis. All values are reported as means ± SE. The statistical significance of differences in mean values between the four animal groups was assessed by two-way ANOVA. A \( P \) value < 0.05 was considered statistically significant.

RESULTS

Metabolic characteristics and body weight of the experimental animals: effect of ANG-(1–7) treatment. At the end of a 6-wk high-fructose diet feeding, body weights were similar.
between standard chow-fed rats and FFR (Table 1). Glycemia remained unaltered after fructose overload (Table 1). Circulating insulin levels were significantly higher in the group consuming high-fructose diet than in the standard chow-fed group (Table 1). Remarkably, circulating insulin levels in FFR that were chronically treated with ANG-(1–7) were not statistically different from those displayed by the control group at the end of the study (Table 1). In control animals, treatment with ANG-(1–7) did not significantly alter circulating glucose or insulin circulating levels (Table 1). This results agree with our previous report (12) showing that chronic infusion of ANG-(1–7) improves insulin resistance in this animal model.

SBP and parameters of cardiac hypertrophy in experimental animals. At the beginning of treatment with ANG-(1–7), FFR that had been exposed to 4 wk of fructose overload displayed a significant increase in SBP compared with the group of rats that received normal diet, (136 ± 3 vs. 109 ± 6; P < 0.05; n = 8).

At the end of the study (week 6), FFR maintained an increase in SBP and displayed a higher heart-to-body weight ratio than the standard chow-fed group (Table 2). FFR that were subjected to a 2-wk treatment with ANG-(1–7) showed a significant reduction in SBP [P < 0.05 vs. FFR treated with ANG-(1–7); n = 8] reaching values not statistically different from those displayed by the standard chow-fed group (Table 2). Interestingly, ANG-(1–7) chronic treatment also restored the heart-to-body weight ratio (Table 2). Microscopic examination of ventricular cross sections revealed an 8% increase in myocyte diameter in FFR (P < 0.05 vs. control sham group; n = 8), which was significantly prevented after ANG-(1–7) chronic treatment (Table 2). All these parameters remained unaltered in standard chow-fed group submitted to a 2-wk treatment with ANG-(1–7) (Table 2).

Interstitial and perivascular fibrosis in the heart. As demonstrated by Masson’s trichrome staining, large areas of interstitial fibrosis were detected in the left ventricles of FFR displaying greater interstitial fibrosis than the standard chow-fed group (Fig. 1; P < 0.01; n = 4). The fibrotic changes in the heart were significantly reduced (46%; P < 0.05; n = 4) in the FFR submitted to a 2-wk treatment with ANG-(1–7) (Fig. 1). No fibrotic areas were observed in control group after ANG-(1–7) treatment (Fig. 1). FFR showed increased perivascular fibrosis in the left ventricle as determined by immunohistochemical analysis of collagen type III deposition (Fig. 2). A 2-wk ANG-(1–7) treatment led to a statistically significant...
reduction in collagen type III immunostained areas (58%; $P < 0.01$; $n = 4$; Fig. 2). No effects were observed in the control group submitted to chronic ANG-(1–7) treatment (Figs. 1 and 2).

Effects of ANG-(1–7) treatment in the phosphorylation degree of MAPKs. Growth-promoting pathways were analyzed in the heart. To this aim, ERK1/2, JNK1/2, and p38MAPK phosphorylation was determined by immunoblotting in rats fed on regular chow or with fructose in drinking water. As shown in Fig. 3, top, a high-fructose diet resulted in an enhancement of phosphorylation levels of ERK1/2, JNK1/2, and p38MAPK (2.1 ± 0.2-, 1.9 ± 0.2-, and 2.5 ± 0.3-fold increase over control group, respectively; $P < 0.05$; $n = 4$). Noticeably, chronic treatment with ANG-(1–7) induced a significant reduction in the phosphorylation degree of all analyzed proteins. No effects were observed in the control group submitted to chronic ANG-(1–7) treatment (Fig. 3, A–C, top). As shown in Fig. 3, changes in phosphorylation level were not a consequence of changes in the abundance of the signaling proteins analyzed (Fig. 3, A–C, bottom).

Effects of ANG-(1–7) treatment on SHP-1 and SHP-2 cardiac activity. To determine SHP-1 and SHP-2 activities in the heart, equal amounts of solubilized proteins were subjected to immunoprecipitation with antibodies to anti-SHP-1 and anti-SHP-2, respectively. Tyrosine-phosphatase activity was evaluated as described in Methods. At the end of the study, cardiac SHP-1 activity was reduced in FFR (27% vs. control animals; $P < 0.05$; $n = 4$; Fig. 4A). FFR that received ANG-(1–7) displayed a cardiac activity of SHP-1 that was undistinguishable from that detected in the control (untreated) group (Fig. 4A). As detected by immunoblotting, the increased SHP-1 phosphatase activity was not due to changes in protein abundance (Fig. 4B). No differences were detected in either SHP-2 cardiac activity (Fig. 4C) or SHP-2 protein abundance in any of the studied groups of animals (Fig. 4D).

Plasma and cardiac ANG II levels of experimental animals. As shown in Table 1, a high-fructose diet caused an increase in ANG II plasma levels (FFR: 215 ± 32 pmol/l) compared with the control group (control: 94 ± 18 pmol/l; $P < 0.05$; $n = 8$; Table 1). ANG II plasma levels remained unaltered after the ANG-(1–7) treatment. Similar results were observed when ANG II local levels were measured in heart sections by immunohistochemistry. In this regard, FFR displayed a remarkably higher immunostained ANG II area in the heart compared with the control group (−6-fold increase; $P < 0.01$; $n = 4$; Fig. 5). Chronic treatment with ANG-(1–7) did not modify ANG II cardiac levels (Fig. 5).

AT1R and Mas receptor abundance in heart of experimental animals. To determine if changes observed in ANG II levels were accompanied by alterations in ANG II-specific receptors or Mas receptor immunostaining levels, heart sections were subjected to immunohistochemistry with anti-AT1R or anti-receptor Mas antibodies. No changes in AT1R were observed in FFR (n = 4; Fig. 6). A slight upregulation of cardiac Mas receptor population was found in the heart of FFR ($P < 0.05$ vs. control group; $n = 4$; Fig. 6). The levels of AT1R and Mas receptor remained unaltered after chronic ANG-(1–7) treatment (Fig. 6). AT1R and Mas receptor abundance in the heart was determined by subjecting heart homogenates to Western blotting analysis. Data obtained in these experiments confirmed data from immunohistochemistry (Fig. 6). No changes in cardiac AT2 receptor immunostaining or abundance were detected (data not shown).

**DISCUSSION**

The renin-angiotensin system is known to play an important role in insulin resistance and diabetes, and it has a critical role in the development of cardiovascular diseases. ANG II is an...
important mediator of these adverse processes (34–37). Once ANG II binds to the AT1R, it activates a series of signaling cascades, which in turn activate serine/threonine kinases such as MAPKs (including ERK1/2, p38MAPK, and JNK1/2) that are implicated in inflammation, cell growth, and hypertrophy. The MAPK pathway has been recognized as a primarily response to growth factors, inflammatory cytokines, and mitogens (26). ERK1/2 can be stimulated upon G protein-coupled

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Fig. 5. Representative sections showing positive immunostaining for ANG II in the left ventricle (original magnification = ×400). Cardiac tissue shows undetectable areas of positive staining for ANG II in control sham group (A) and control group treated with ANG-(1–7) (B). In fructose sham group (C) and fructose group treated with ANG-(1–7) (D), left ventricle sections show extended areas of positive staining for ANG II indicated by arrows. Cardiac sections from FFR were incubated with either PBS (E) or with anti-ANG II antibody previously blocked by preabsorption with ANG II (F). Scale bar = 50 μm. Bars represent the percentage of immunostaining for ANG II in the heart of all groups (G). Data are means ± SE. †P < 0.01 vs. control sham group by two-way ANOVA (n = 4).
INHIBITION OF CARDIAC REMODELING BY ANG-(1–7)

A)

AT1R

Control – sham

Control – Ang-(1-7)

Fructose – sham

Fructose – Ang-(1-7)

B)

AT1R cardiac levels

(Percentage of

immunostaining/mm²)

Control sham

Control ANG-(1-7) sham

Control Fructose ANG-(1-7)

C)

IB: AT1R

IB: β-Actin

D)

Mas receptor

Control – sham

Control – Ang-(1-7)

Fructose – sham

Fructose – Ang-(1-7)

E)

Mas receptor cardiac levels

(Percentage of

immunostaining/mm²)

Control sham

Control ANG-(1-7) sham

Control Fructose ANG-(1-7)

F)

IB: Mas receptor

IB: β-Actin
receptor occupation by hormones such as ET-1 or ANG II in cardiac myocytes (43). Through its interactions with the AT1R, ANG II increases fibroblast gene expression (including collagen type I and III), fibroblast density and proliferation, and myocyte hypertrophy, all of which are hallmarks of myocardial fibrosis and remodeling (13, 22, 43).

Besides its ability to activate ERK1/2, ANG II is also known to stimulate the activity of JNK1/2 and p38MAPK (3, 23, 32, 49). Moreover, stretch of cardiomyocytes, which is a primary stimulus for cardiac hypertrophy, causes activation of MAPks (38). Thus increased levels of activated MAPks present in high-fructose fed rats could be considered as an important event for the cardiac hypertrophy detected in these animals.

Several studies (14, 15, 19, 27, 46) have shown that ANG-(1–7) has cardioprotective effects by preventing cardiac fibrosis and hypertrophy. The cellular mechanisms responsible for the ANG-(1–7)-dependent reduction in cardiomyocyte growth appear to involve a decrease in serum-stimulated MAPks activity (45). These results agree with studies performed in vascular smooth muscle cells as well as in rat proximal tubular cells showing that attenuation of ANG II-induced ERK1/2 is one of the mechanisms by which ANG-(1–7) inhibits cell growth and proliferation (42, 44). Indeed, in a previous work, we (11) demonstrated that ANG-(1–7) inhibited ANG II-induced acute activation of ERK1/2 in the heart. Accordingly, it was also demonstrated that ANG-(1–7) blocks high glucose-induced p38MAPK activation (9).

A high-fructose diet is known to induce a mild hypertensive state in rats with increased ANG II plasma levels that may account for the cardiac remodeling present in this animal model (18, 21). Interestingly, ANG-(1–7) was able to improve both the hypertrophic and fibrogenic processes presented in FFR. A significant reduction of interstitial fibrosis areas was observed after chronic treatment with ANG-(1–7). However, this reduction was partial and did not reach control sham values. In addition, ANG-(1–7) induced a marked reduction of perivascular fibrosis (detected as collagen type III deposition) in the heart.

Importantly, ANG-(1–7) reduced insulin plasma levels, which could have contributed to the beneficial effects observed after this treatment. A key feature of insulin resistance is the specific impairment in phosphatidylinositol 3-kinase-dependent signaling pathways, whereas other insulin-signaling branches including Ras/MAPK-dependent pathways are unaffected (5, 20). This has important pathophysiological implications because metabolic insulin resistance is usually accompanied by compensatory hyperinsulinemia to maintain euglycemia. In the vasculature and also in target tissues such as the heart, hyperinsulinemia will overdrive unaffected MAPK-dependent pathways leading to an imbalance between phosphatidylinositol 3-kinase- and MAPK-dependent functions of insulin, promoting hypertrophy (28). An additional benefit that arose from ANG-(1–7) therapy was a significant reduction in systolic blood pressure in this animal model, which could have contributed to the observed amelioration of cardiac morphological alterations.

Another major finding that should be pointed out was that ANG-(1–7) attenuated three important growth-promoting pathways in the heart of insulin-resistant rats. After chronic treatment with ANG-(1–7), basal phosphorylation levels of ERK1/2, JNK1/2, and p38MAPK were reduced to control values. Thus ANG-(1–7)-induced inhibition of MAPK pathways could explain, at least in part, the beneficial effects exerted by this hormone in heart tissue.

However, from the available data it is not possible to conclude whether the observed beneficial effects exerted by ANG-(1–7) are direct (through its own receptor attenuating growth-promoting pathways) or indirect (by decreasing insulinemia and blood pressure).

In good agreement with the current results, in a recent study, Mercure et al. (27) have shown that transgenic mice with chronic selective elevation of ANG-(1–7) in the heart are protected from ventricular hypertrophy and fibrosis in response to ANG II infusion. Unlike what was detected in the present study, the protective effect of ANG-(1–7) did not correlate with a reduction of systolic blood pressure (27). There were also some interesting discrepancies regarding the modulation of downstream molecular markers of cardiac remodeling, since this inhibitory effect of ANG-(1–7) was accompanied by a reduction of p38MAPK activation, but targeted elevation of ANG-(1–7) in the heart did not block the stimulation of ERK1/2 phosphorylation (27). The tyrosine phosphatases SHP-1 and SHP-2 negatively influence AT1R downstream signaling molecules, such as MAPKs, through dephosphorylation and thereby inhibiting growth and inflammatory signaling activated by ANG II (4, 6). Several lines of evidence suggest that ANG-(1–7) may antagonize growth-promoting pathways through activation of SHP-1 (9) and SHP-2 (39). In line with these reports, by the end of the treatment, FFR exhibited a reduction of SHP-1 activity that was not exhibited by the group of FFR that received ANG-(1–7). SHP-1 leads to dephosphorylation of MAPKs and could represent an inhibitory mechanism of growth-promoting pathways. This is an additional difference with the results found in the study of Mercure et al. (27), since chronic expression of ANG-(1–7) selectively in the heart leads to increased ventricular expression of SHP-2 with or without ANG II infusion. The mentioned disparity between the two models could be attributed to differences in the circulating levels of both ANG II and ANG-(1–7) and the metabolic profiles of the animal models, which are very different since FFR are insulin resistant, and this could affect substrate delivery to the heart. The duration of exposure to high levels of ANG-(1–7) may be an additional difference between these two models. Exposure to high levels of ANG-(1–7) in the heart of transgenic mice begins at birth, and FFR used in the current study were treated only for 2 wk with this hormone.

Fig. 6. Representative sections showing positive immunostaining for ANG II type 1 receptor (AT1R) and Mas receptor in the left ventricle (original magnification ×400). Areas of positive staining for AT1R (A) and Mas receptor (D) are indicated by arrows. Scale bar = 50 μm. Bars represent the percentage of immunostaining for AT1R (B) and Mas receptor (E) in the heart of all groups of animals. Western blot analysis was performed to determine AT1R and Mas receptor protein abundance. Solubilized heart proteins were subjected to immunoblotting with anti-AT1R (C, top) and anti-Mas receptor (F, top). To evaluate protein load, membranes were reprobed with an anti-β-actin antibody. Data are means ± SE. *P < 0.05 vs. control sham group by two-way ANOVA (n = 4).
FFR displayed increased plasma and cardiac ANG II levels that remained unaltered after a 2-wk treatment with ANG-(1–7), indicating that positive effects observed after chronic ANG-(1–7) administration were not due to a reduction in ANG II levels. Furthermore, we analyzed ANG II-specific receptors; FFR showed normal levels of cardiac AT1R and increased Mas receptor protein content. Taking into account that the Mas receptor participates in glucose and lipid metabolism and that Mas receptor deficiency leads to a metabolic syndrome-like state (41), the observed upregulation of Mas receptor abundance could represent a compensatory mechanism to the alterations induced by high-fructose diet in rats. Importantly, chronic ANG-(1–7) treatment did not modify either AT1R or Mas receptor abundance in the heart. However, all these observations do not preclude that the cardiac effects of ANG-(1–7) could involve modulation of the binding characteristics and/or actions of these receptors; indeed, it has been well documented that ANG-(1–7) is an important modulator of the renin-angiotensin system and attenuates the effects originated by ANG II (47).

Recent research has underscored the importance of heightened activation of the renin-angiotensin-aldosterone system and sympathetic nervous system, oxidative stress, inflammation, and mitochondrial functional abnormalities in promoting insulin resistance (50). In addition to the negative effects of these factors on insulin metabolic signaling in conventionally insulin-sensitive tissue, such as skeletal muscle, there is a simultaneous negative effect on metabolic signaling in cardiovascular tissue. These negative effects include reduced insulin stimulation of endothelial cell nitric oxide production and increased nitric oxide destruction with resulting endothelial dysfunction and hypertension (50). In this regard, our current study showing the concomitant amelioration of insulin resistance, reduction of systolic blood pressure, and improvement of cardiac remodeling after chronic ANG-(1–7) treatment shows that this hormone could have a protective role in the cardiac remodeling syndrome.

In conclusion, we have shown that chronic administration of ANG-(1–7) decreases systolic blood pressure, reduces circulating insulin levels, and improves cardiac morphological alterations in the heart of FFR. ANG-(1–7) was able to reduce insulin resistance differentially affecting the positive glucose-induced signaling in proximal tubular cells. Nephrol Dial Transplant 24: 1766–1773, 2009.


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See references for full citations and details.

References:


