Angiotensin(1-7) Blunts Hypertensive Cardiac Remodeling by a Direct Effect on the Heart

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Abstract—Angiotensin-converting enzyme 2 (ACE2) converts the vasopressor angiotensin II (Ang II) into angiotensin (1-7) [Ang(1-7)], a peptide reported to have vasodilatory and cardioprotective properties. Inactivation of the ACE2 gene in mice has been reported by one group to result in an accumulation of Ang II in the heart and an age-related defect in cardiac contractility. A second study confirmed the role of ACE2 as an Ang II clearance enzyme but failed to reproduce the contractility defects previously reported in ACE2-deficient mice. The reasons for these differences are unclear but could include differences in the accumulation of Ang II or the deficiencies in Ang(1-7) in the mouse models used. As a result, the roles of ACE2, Ang II, and Ang(1-7) in the heart remain controversial. Using a novel strategy, we targeted the chronic overproduction of either Ang II or Ang(1-7) in the heart of transgenic mice and tested their effect on age-related contractility and on cardiac remodeling in response to a hypertensive challenge. We demonstrate that a chronic accumulation of Ang II in the heart does not result in cardiac contractility defects, even in older (8-month-old) mice. Likewise, transgenic animals with an 8-fold increase in Ang(1-7) peptide in the heart exhibited no differences in resting blood pressure or cardiac contractility as compared to age-matched controls, but they had significantly less ventricular hypertrophy and fibrosis than their nontransgenic littermates in response to a hypertensive challenge. Analysis of downstream signaling cascades demonstrates that cardiac Ang(1-7) selectively modulates some of the downstream signaling effectors of cardiac remodeling. These results suggest that Ang(1-7) can reduce hypertension-induced cardiac remodeling through a direct effect on the heart and raise the possibility that pathologies associated with ACE2 inactivation are mediated in part by a decrease in production of Ang(1-7).

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Key Words: angiotensin ■ hypertension ■ hypertrophy ■ remodeling

Angiotensin-converting enzyme 2 (ACE2), a homologue of the ACE enzyme1,2 expressed primarily in the vascular endothelium of the heart, kidney, and forebrain,3 removes a single amino acid from the carboxy-terminus of angiotensin II (Ang II) to generate angiotensin(1-7) [Ang(1-7)]. Inactivation of ACE2 in mice has been reported to result in an age-dependent cardiac contractility defect associated with a decrease in systolic blood pressure in older (6-month-old) male mice, an upregulation of hypoxia-associated genes in the heart, and an increase in accumulation of Ang II in cardiac tissue.4 These findings led the authors to suggest that ACE2 functions as a clearance enzyme for Ang II in the heart and that the accumulation of Ang II results in the observed cardiac defects over time. This possibility was supported by the finding that the compound inactivation of both ACE2 and ACE (thereby rendering the animals unable to synthesize Ang II) corrects the cardiac contractility defect seen in the older ACE2 knockout male mice.4 However, ACE2 inactivation would also be expected to reduce Ang(1-7) content in the heart and compound inactivation of both ACE and ACE2 could lead to restoration of Ang(1-7) levels by causing an accumulation of Ang I. Thus, it is not clear whether the contractility defects reported by Crackower et al4 result from an accumulation of cardiac Ang II or a deficit in Ang(1-7) production.

More recently, Gurley et al5 reported that inactivation of ACE2 resulted in a modest increase in basal blood pressure in the C57BL/6 mouse strain that correlated with a deficiency in plasma Ang II clearance after bolus administration, confirming that ACE2 can function as an Ang II clearance enzyme. Notably, there was no blood pressure effect of ACE2 inactivation in the 129/SvEv strain of mice, and no cardiac contractility defects were detected in any of the ACE2 knockout mice. The reason for the differences in the findings by these 2 groups are not entirely clear but could be attributed to varying genetic backgrounds in the mice used or effects of ACE2 inactivation in noncardiac tissues or in other environmental or physiological variables. As a result, it is still
unclear whether cardiac dysfunction can result from a chronic increase in Ang II or the loss of a protective effect of Ang(1-7) specifically in the heart.

In the current study, we have used a novel technique to directly test for the role of the 2 angiotensin peptides on the heart. Our results show that Ang(1-7) can protect the heart from hypertension-induced remodeling and provide the first evidence to our knowledge for a direct cardiac effect of Ang(1-7) in vivo.

Materials and Methods

Animals

The mice used in this study were housed in a 12/12 hour light/dark cycle with free access to normal mouse chow and water. All of the experiments described herein were approved by the institutional Animal Ethics Committee and are in compliance with guidelines issued by the Canadian Council on Animal Care.

Production of Transgenic Mice

Production of mice with chronic elevations of cardiac angiotensin II (Ang II) in the FVB/N line has been previously described. The mice used in this article correspond to the 1B founder line and have been used in this article. Generation of the mice expressing Ang(1-7) in the heart was performed by expressing a fusion protein under the control of the mouse histone H4 (a-MHC, a gift from Dr. Jeffrey Robbins, University of Cincinnati). Transgenic mouse generation was performed using standard protocols. Line VII-7 was derived from injection of inbred C57BL/6 embryos, whereas the VII-7b and VII-7c lines were derived from C57BL/6 X C57 FL embryos injections. All of the physiological characterization was performed in inbred mice (line VII-7) or in mice that had been backcrossed for >6 generations (F6) onto the C57BL/6 (Harlan, Toronto, Canada) strain. Genomic integration of the transgene was determined by polymerase chain reaction analysis of DNA obtained from tails that underwent biopsies.

Transgene Expression and Peptide Production

Transgene expression was confirmed by extracting total tissue RNA by the guanidinium isothiocyanate method and performing an RNase protection assay on 10 μg of total RNA for the transgene and histone H4 (an internal control) as previously described. Production of Ang(1-7) in tissues of transgenic mice was measured by radioimmunoassay as described by Botelho et al.6 Briefly, animals were euthanized by CO2 inhalation and tissues were rapidly extracted and homogenized in 5 mL of 4 mol/L guanidinium hydrochloride. The lysate was cleared by centrifugation (10000g, 15 min) and peptides were extracted using a High Flow Bond Elut LRC-C18 cartridge (Varian, Harbor City, Calif). Sequential washes with 20 mL of 99.9% acetoni0l/0.1% HFB, 20 mL of 0.1% HFB, 3 mL of 0.1% HFB containing 0.1% fat-free bovine serum albumin, 10 mL of 10% acetonitrile/0.1% HFB, and 3 mL of 0.1% HFB were used to activate the columns. After sample application, the columns were washed with 20 mL of 0.1% HFB and 3 mL of 20% acetonitrile/0.1% HFB. The adsorbed peptides were eluted with 3 mL of 99.9% acetonitrile/0.1% HFB into polypropylene tubes rinsed with 0.1% fat-free bovine serum albumin. After evaporation, Ang(1-7) levels were measured by high-performance liquid chromatography-coupled radioimmunoassay as described by Botelho et al.

Hypertensive Challenge

Osmotic minipumps (Alza Corp, Palo Alto, Calif) were implanted subcutaneously in anesthetized (2% isoflurane) 12-week-old male transgenic and control mice for the administration of Ang II (350 ng/kg/min) or saline. Animals were allowed to recover for 1 week, after which blood pressure was measured for 9 days and the animals were subjected to echocardiography. After physiological characterization (19 days after minipump implantation), the mice were euthanized and the hearts were collected for histology and assessment of ventricular hypertrophy.

Physiological Characterization

Cardiac geometry and function were evaluated with a Sonos 5500 (Philips) echo Doppler equipped with a 15- to 6- to 6 to 15-MHz ultraband Intraoperative Linear Array probe. All studies were performed on lightly anesthetized (2% isoflurane, 5 min) mice maintained at 37°C. M-mode-derived measures of left ventricle (LV) area were obtained, using the leading edge-to-leading-edge convention adopted by the American Society of Echocardiography.10 Temporal changes between LV systolic diameter (LVD) and LV diastolic diameter were used for the calculation of percent shortening fraction, as follows [(LVD diameter – LVD)/LVD diameter]×100. Three heart beats were averaged for each measurement.

Systolic blood pressure was measured by computer-automated tail-cuff plethysmography (model BP-2000; Visitech Systems, Apex, NC). Briefly, mice were trained to the apparatus for 9 continuous days and measurements were recorded only for the last 4 days. Mean values for these last 4 days were calculated for each animal.

Assessment of Cardiac Remodeling

Ventricular hypertrophy was assessed by measuring the ratio of combined ventricular wet weight to total body weight (body weight did not vary significantly between the animal groups studied). For determination of cardiomyocyte hypertrophy and cardiac collagen content, mice were anesthetized by intraperitoneal injection of 3 mg pentobarbital sodium (MTC Pharmaceuticals, Cambridge, Ontario). Blood was chased from major vessels by whole-body perfusion of saline solution through the heart, followed by in situ organ fixation using 40 mL Bouin fixative solution (0.9% picric acid, 10% formaldehyde, and 5% glacial acetic acid). Organs were quickly removed and postfixed for 5 hours. Fixed tissue was stored in 70% ethanol at 4°C until analyzed.

To determine myocyte cross-sectional area, heart sections were stained with hematoxylin and eosin and examined with a Zeiss Axioskop at a magnification of 60×. To ensure that the sections were perpendicular, only myocytes in which the nucleus was centrally located within the cell were used. The circumference of the myocytes was traced using imaging software (Northern Eclipse 7.0; EM-PIX Imaging Inc), and the myocyte cross-sectional area (μm2) was determined by averaging the calculated area of 180 to 200 individual cardiomyocytes within the ventricular free wall over 5 to 6 sections per animal.

To quantify cardiac fibrosis, sections were stained with Sirius Red F3BA (0.5% in saturated aqueous picric acid; Sigma-Aldrich, Oakville, Ontario) for assessment of interstitial and perivascular collagen content, as previously described.11 Quantification of fibrosis was performed using an image analysis system (Northern Eclipse 7.0; EM-PIX Imaging Inc). Seven fields were analyzed on 3 independent sections per mouse to determine the collagen area/total area. A single investigator unaware of the experimental groups performed the analysis.

Modulation of Cardiac RNA Expression

Total RNA was isolated from mouse ventricles with TRizol (Invitrogen). Quantitative reverse transcriptase polymerase chain reaction was performed on cDNA generated with the Omniscript RT Kit (QIAGEN, Inc) with the Quantitect SYBR Green polymerase chain reaction kit (QIAGEN Inc) in a MX3005 real-time polymerase chain reaction machine (Stratagene). The oligonucleotides were designed to have a melting temperature of 60°C and were used with an annealing temperature of 58°C. The oligonucleotides used for quantitative polymerase chain reaction were 5'-CCGATAGATCGCCTTGGTCCGTG-3' (forward) and 5'-GGTGAATCTCACTGGATTTTGCA-3' (reverse) for brain natriuretic peptide; 5'-CCGCTCTTGAGGACCAACTG-3' (forward) and 5'-CCGCAAAACAGAGGACATG-3' (reverse) for brain natriuretic peptide; 5'-TGGTGGGAGATTGGAAAC-3' (forward) and 5'-
GATGATGCAGGTGACTTTGG-3
H11032
(reverse) for the Ang II AT1 receptor
detects both AT1a and AT1b); 5
H11032-GTCATTGACCTGGCACTTCC-3
H11032 (forward) and 5
H11032-TGGCTAGGCTGATTACATGC-3
H11032 (reverse) for the
Ang II AT2 receptor; 5
H11032-TGAGTGGCTGTCTTTTGACG-3
H11032 (forward)
and 5
H11032-CGCACACAGCAGTTCTTCTC-3
H11032 (reverse) for transforming
growth factor beta (TGF beta 1); and 5
H11032-TCTGGGCAAGGAGA-
GATTTG-3
H11032 (forward) and 5
H11032-CCGCCAAACTTCTTGGATTC-3
H11032 (reverse) for 40S ribosomal protein S16. Expression of the corresponding
RNA is shown relative to S16, arbitrarily set to 1.0 for control.

Western Blotting Analysis
Mice were euthanized by CO2 inhalation and the hearts were rapidly
excised and frozen in liquid nitrogen. Frozen hearts were homoge-
nized in lysis buffer (50 mmol/L Tris/HCl, pH 7.4; 5 mmol/L EGTA
and 2 mmol/L EDTA, 5% Triton 100, 0.1 mmol/L PMSF, 1 mmol/L
pepsatin A, 1 mmol/L leupeptin, and 1 mmol/L aprotinin). Thirty
micrograms of protein were separated by electrophoresis on a 10%
polyacrylamide gel and probed for content of GAPDH, SHP-1, and
SHP-2, as well as phosphorylated c-Src, p38MAPK, and ERK1/2 by
standard methodologies (see online supplement for expanded meth-
dods at http://circres.ahajournals.org/cgi/content/full/CIRCRESAHA.108.184911/DC1).

Phosphatase Assay
Protein tyrosine phosphatase assays for SHP-1 and SHP-2 were
performed by a modification of previously published methods12 (see
online supplement at http://circres.ahajournals.org/cgi/content/full/CIRCRESAHA.108.184911/DC1).

Statistical Analysis
Unpaired t test (Figure 1–3) and 1-way ANOVA with Bonferroni

Responsibility
The authors had full access to the data and take responsibility for its
integrity. All authors have read and agree to the article as written.
Results

Cardiac Ang II and Contractility

Transgenic mice expressing an Ang II-generating fusion protein in the heart exhibit a chronic 20- to 50-fold increase in cardiac Ang II. For the current study these mice were crossed for 8 generations into the C57BL/6J background. To test whether this local increase in cardiac Ang II would lead to age-dependent contractility defects, 8-month-old male transgenic mice and control littermates were subjected to echocardiography. Results show no discernible difference in heart rate, blood pressure, chamber dimensions (not shown), or contractility (Figure 1) between transgenic and control mice (fractional shortening 37.8±2.2% control, 37.2±2.4% Ang II transgenic). These results demonstrate that chronic increases of Ang II in the heart are not sufficient to cause contractility defects, even in older mice.

Generation of Mice With Chronic Increases in Cardiac Ang(1-7)

To test whether cardiac Ang(1-7) could play a cardioprotective role, we used a similar technology to generate transgenic mice expressing an Ang(1-7)-releasing fusion protein under the control of the cardiac-specific α-MHC promoter (Figure 2A). Three independent founder lines were obtained that show the expected expression of the transgene in the heart (Figure 2B) and a lower and variable level of expression of the transgene in the lung, as is commonly reported for this promoter. We have not detected any underrepresentation or any increase in sudden death in any of the lines of mice overexpressing Ang(1-7) in the heart over the past 9 generations, although we were unable to generate offspring from line VII-7B (data not shown). These results demonstrate that overproduction of Ang(1-7) in the heart is not lethal. Using line VII-7, we measured tissue-specific production of the Ang(1-7) peptide in heart, lung, blood, and kidney (Figure 3). As expected, the expression of the transgene led to 8-fold increase in Ang(1-7) in the heart. A 2- to 3-fold increase of Ang(1-7) was also detected in the lung of transgenic mice that may be attributable to the previously reported residual activity of the promoter in pulmonary vessels branching from the heart, and there was a nonsignificant trend toward an increase in the kidney although the transgene was not expressed in this tissue (Figure 2B). Notably, there was no detectable increase in the content of Ang(1-7) in the blood of transgenic animals, thereby demonstrating that we were successful in deriving a mouse model of cardiac overexpression of Ang(1-7).

Physiological Characterization of MHC Ang(1-7) Mice

Because Ang(1-7) has been reported to have vasodilatory and cardiac effects, we characterized MHC–Ang(1-7) (VII-7) mice for heart rate, blood pressure, and cardiac function. Results (Table) demonstrate that chronic overproduction of Ang(1-7) in the heart does not affect blood pressure, heart rate, cardiac geometry, or contractility (as measured by chamber volume and fractional shortening). Thus, under normal hemodynamic conditions, cardiac Ang(1-7) does not affect cardiac function. To determine if cardiac Ang(1-7) might provide protection against cardiac remodeling associated with hypertension, we chronically infused control and MHC Ang(1-7) (VII-7) mice with pressor doses of Ang II and measured cardiovascular parameters (Figure 4). As expected, chronic infusion of Ang II for a period of 19 days led to a
A significant increase in systolic blood pressure that was equivalent in transgenic and control mice (Figure 4A). Likewise, both groups showed a trend toward a decrease in fractional shortening that did not reach statistical significance (Figure 4B). In contrast, although control mice responded to the Ang II infusion with a 20% increase in ventricular weight, transgenic VII-7 mice had a significantly diminished hypertrophic response (Figure 4C). These same differences were reflected in the size of cardiomyocytes (Figure 4D). Likewise, cardiac fibrosis increased by >2-fold in control mice infused for 19 days with Ang II while the fibrotic response was approximately halved in transgenic VII-7 mice (Figure 4E). In conclusion, chronic production of Ang(1-7) in the heart blunts cardiac remodeling in response to Ang II-induced hypertension.

**Molecular Markers of Cardiac Remodeling**

In an effort to better define the site of action of Ang(1-7) in cardioprotection, the expression of markers and mediators of...
Ang II infusion (Figure 5C). In contrast, we found no effect of the transgene on expression of AT1 (AT1a and AT1b) or AT2 receptors in either the hearts or the kidneys of mice (see online supplement for Figure I at http://circres.ahajournals.org/cgi/content/full/CIRCRESAHA.108.184911/DC1). These results confirm that the effect of Ang(1-7) on cardiac remodeling is reflected by changes at the molecular level but that they are not explained by an alteration in the expression of angiotensin receptor levels.

Ang(1-7) Selectively Modulates Growth Signaling Pathways in Ang II-Infused Mice

To investigate the mechanisms by which local Ang(1-7) reduces hypertension-induced cardiac remodeling, we assessed a number of intracellular signaling pathways known to be associated with hypertrophic remodeling, including c-Src, p38, and ERK1/2 mitogen-activated protein kinase (MAPK) (Figure 6). Chronic production of Ang(1-7) in the hearts of mice had no effect of the basal level of phosphorylation of these signaling kinases, as compared to control animals. In addition, the phosphorylation of all of these signaling molecules was increased in response to Ang II infusion in control mice as has been previously reported. In contrast, mice expressing Ang(1-7) in the heart showed a significant decrease in phosphorylation of c-Src (Figure 6A) and p38 kinase (Figure 6B) seen after Ang II infusion, whereas the increase in ERK1/2 phosphorylation was unaffected by expression of the transgene (Figure 6C). These results demonstrate a selective effect of Ang(1-7) on intracellular signaling pathways that mediate cardiac remodeling.

Ang(1-7) Modulates SHP-2 Activity in Ang II-Infused Mice

To better understand how Ang(1-7) selectively modulates the phosphorylation status of signaling kinases, we assayed protein tyrosine phosphatase activity in the heart. Whereas Ang II infusion tended to decrease cardiac SHP-1 protein tyrosine phosphatase activity in both control and transgenic mice (Figure 7A), SHP-2 activity decreased in the hearts of control mice but not in those of transgenic mice treated with Ang II (Figure 7B). To investigate the mechanisms by which cardiac Ang(1-7) modulates SHP-2 activity, we assessed SHP-2 expression (Figure 7C). Transgenic VII-7 mice displayed increased ventricular expression of SHP-2 with or without Ang II infusion. These results suggest that Ang(1-7) causes an increase in SHP-2 content in the heart that correlates with a maintenance of SHP-2 activity in the context of a hypertensive challenge.

Discussion

Inactivation of the ACE2 gene in mice leads to Ang II accumulation in tissues that has been variously reported to be associated with hypertension and age-related decreases in cardiac contractility by different investigators. Conversely, targeted overexpression of ACE2 in the heart results in heart block, ventricular tachycardia, and sudden death associated with a downregulation of connexin 40 and connexin 43 in 2-month-old mice. Because ACE2 mediates the conversion of Ang II to Ang(1-7) and would therefore lead to changes in the levels of both peptides with its modulation, we used a novel...
approach to test for the effect of selective increases of each of these peptides individually in the hearts of transgenic mice.

Our results show that mice with a chronic and very large (20- to 50-fold) increase in Ang II exclusively in the heart did not exhibit the decreases in cardiac contractility previously reported in mice deficient for ACE-2,4 even at advanced age, making it unlikely that this cardiac phenotype is attributed to an accumulation of Ang II in the heart. This possibility is further supported by our finding that local production of Ang(1-7) in the heart can provide protection against cardiac remodeling associated with hypertension. The possibility that Ang(1-7) is important for heart function is also supported by other gene manipulation studies in mice. The complete inactivation of the RAS by knocking out angiotensinogen also leads to impaired systolic function in mice,19 suggesting that the lack of some product of the RAS is responsible. Furthermore, Santos et al20 demonstrated that mice defective for Mas, the putative Ang(1-7) receptor, exhibit decreased fractional shortening. The decreased cardiac contractility seen in this latter study was obvious in normotensive mice and was attributed to increased overproduction of certain extracellular matrix proteins in the hearts of Mas-deficient mice.20 Although overproduction of Ang(1-7) in the heart did not lead to an apparent decrease in collagen content in the normotensive mice in our studies, our results do confirm a role for Ang(1-7) in the control of cardiac collagen content in hypertension. It is also notable that the chronic overproduction of Ang(1-7) we achieved in our studies did not result in any detectable adverse effects on the heart and did not decrease survival of our transgenic mice.

Whereas our current results support a direct effect of Ang(1-7) on the heart, the target cell is more difficult to identify because the approach we used is likely to lead to a general increase in Ang(1-7) in the interstitial spaces around cardiomyocytes. It seems quite likely, however, that the cardiomyocyte is a primary target since the antihypertrophic effect of local Ang(1-7) is mirrored by effects on cardiomyocyte size (Figure 4). Ang(1-7) selectively binds to cardiomyocytes in vitro and in vivo, and this binding disappears in Mas receptor-deficient mice.20,21 It is less clear how Ang(1-7) acts to reduce hypertensive cardiac fibrosis because there has been no direct demonstration of Ang(1-7) receptors on fibroblasts. However, by using mice chimeric for Ang II AT1 receptor deficiency, Matsusaka et al22 elegantly demonstrated that cardiac fibroblast proliferation in response to a hypertensive challenge is orchestrated by the cardiomyocytes, raising the possibility that Ang(1-7) interferes with the signaling mechanism before it leaves the cardiomyocyte.

Interestingly, increasing cardiac Ang(1-7) led to a slight (1.5×), but significant increase in ventricular atrial natriuretic peptide gene expression in the normotensive animals (Figure 5A). In addition to being a marker of hypertrophy, we have previously obtained evidence that ventricular atrial natriuretic peptide may also play a role in cardioprotection through stimulation of intracellular cGMP accumulation in cardiomyocytes.23 Nevertheless, we found no increase in cardiac cGMP content in the hearts of mice producing Ang(1-7) locally (data not shown). Rather, it appears that Ang(1-7) selectively modulates MAPK signaling in the heart.

Cardiac hypertrophy can result in response to a number of stimuli, including hormones, cytokines, and biomechanical stress (stretch). All of these stimuli result in enhanced MAPK signaling in cardiomyocytes and, through the activation of kinase cascades, lead to the ultimate phosphorylation of terminal kinases such as ERK, p38, and JNK kinases.17 These, in turn, regulate cardiomyocyte gene expression through selective transcription factor phosphorylation. Biomechanical stress signals can also be transduced through integrin-mediated activation of focal adhesion kinase–Src kinase complex leading to the activation of small GTPases such as Ras and Rho.24 We tested whether Ang(1-7) might provide protection from cardiac remodeling by modulating ≥1 of these hypertrophic signaling pathways. Our findings demonstrate that Ang(1-7) modulates some (c-Src and p38 MAPK) but not all (ERK1/2) growth signaling pathways in the heart in response to hypertension. The inhibitory effect of Ang(1-7) on p38 MAPK activation might also explain the apparent decrease in hypertension-induced transforming growth factor β-1 expression seen in the transgenic mice (Figure 5C) because p38 is one of the known mediators of both transforming growth factor β-1 expression and activation.25 The lack of effect on ERK1/2 signaling is surprising because its phosphorylation is closely correlated with hypertrophic remodeling. However, Purcel et al26 recently reported that mice with targeted inactivation of the ERK1/2 genes showed no reduction in the development of pathological hypertrophy. This led the authors to suggest that ERK1/2 may actually play a protective role in response to pathological stimuli. A local increase in Ang(1-7) also correlates with an increase in the ventricular content of SHP-2 protein tyrosine phosphatase and an increase in its activity in the face of Ang II infusion. This increase in phosphatase activity may explain the reduced phosphorylation of c-src and p38 MAPK, and this will remain an interesting area for further study.

### Table. Physiological Characterization of TG Mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Heart Rate, bpm</th>
<th>Systolic BP, mm Hg</th>
<th>%FS, mm</th>
<th>LVDd, mm</th>
<th>LVDs, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>653±13</td>
<td>106.8±2.9</td>
<td>38.1±1.3</td>
<td>4.17±0.09</td>
<td>2.58±0.14</td>
</tr>
<tr>
<td>Ang(1-7) TG (line VII-7)</td>
<td>617±12</td>
<td>113.4±2.8</td>
<td>39.1±1.7</td>
<td>4.12±0.08</td>
<td>2.51±0.08</td>
</tr>
<tr>
<td>Ang(1-7) TG (line VII-7C)</td>
<td>600±12*</td>
<td>95.3±2.9†</td>
<td>38.4±1.8</td>
<td>3.96±0.10</td>
<td>2.45±0.12</td>
</tr>
</tbody>
</table>

Values represent the mean±standard error. N=12 animals/group.

%FS indicates percent fractional shortening; bpm, heart rate in beats per minute; LVDd, left ventricular diastolic diameter; LVDs, left ventricular systolic diameter; TG, transgenic.

*P<0.05 vs control.

†P<0.01 vs control.
Although it is also possible that Ang(1-7) inhibits the Ang II receptor directly to reduce cardiac remodeling resulting from Ang II infusion, we found no evidence that chronic overproduction of Ang(1-7) decreased AT1 receptor expression in the hearts or kidneys of our transgenic mice. It has also been suggested that Ang(1-7) can downregulate Ang II signaling by promoting heterodimerization between the Mas receptor and the Ang II AT1 receptor.27 However, a specific reduction of Ang II signaling by Ang(1-7) is an unlikely explanation for our results for several reasons. First, local production of Ang II in the heart does not result in the cardiac remodeling we see in our Ang II-infused mice,6 suggesting that it is not attributed to a direct effect of Ang II signaling in cardiomyocytes. Thus, a local production of Ang(1-7) in the heart could not reverse remodeling by blocking local Ang II signaling. Second, we had previously demonstrated that circulating Ang(1-7) blunts cardiac remodeling attributed to isoproterenol injection.8 Thus, Ang(1-7) must act to block signaling of >1 type of hypertrophic stimulus, consistent with a role in the signaling pathways common to these stimuli. Finally, Ang(1-7) did not block the stimulation of ERK1/2 phosphorylation, a classical downstream target of Ang II receptor activation in tissue culture. Taken together, our results suggest that Ang(1-7) acts on cardiomyocytes to block signaling pathways that trigger cardiac remodeling in an acute hypertensive model. Whether the Ang(1-7) peptide can also provide cardioprotection from chronic hypertensive heart disease will be a topic of future interest.

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**Disclosures**

None.

**References**


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**EXPANDED METHODS:**

**Western Blotting analysis**
Mice were euthanized by CO₂ inhalation and the hearts were rapidly excised and frozen in liquid nitrogen. Frozen hearts were homogenized in lysis buffer [50 mmol/l Tris/HCl (pH 7.4), 5 mmol/l EGTA and 2 mmol/l EDTA, 5% Triton 100, 0.1 mmol/l PMSF, 1 mmol/l pepstatin A, 1 mmol/l leupeptin and 1 mmol l aprotinin]. Thirty micrograms of protein were separated by electrophoresis on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% skim milk or 1% BSA in Tris-buffered saline solution with Tween for 1 hour at 24°C. Membranes were then incubated with primary antibodies (1:1000) overnight at 4°C. Antibodies were as follows: anti–c-Src (Tyr418) (Biosource International), anti-SHP-2 (Santa Cruz), anti anti–p38 MAPK Thr180/Tyr182), anti-ERK1/2 (Thr202/Tyr204) and anti–SHP-2 (Tyr 580) (the latter 3 from Cell Signaling Technology, Inc). Immunoblots for nonphosphoproteins (c-Src, p38 MAPK, ERK1/2, Akt) or GAPDH (Chemicon) were carried out in the same membranes used as a loading control. After incubation with secondary antibodies, signals were revealed by chemiluminescence, visualized by autoradiography, and quantified densitometrically. Results were normalized by the total protein or GAPDH.

**Phosphatase assay**
Hearts were homogenized in immunoprecipitation buffer without sodium orthovanadate (15 mM KCl, 10 mM HEPES, 2 mM MgCl₂, 0.1% NP40, 1mM PMSF, 1µg/mL aprotinin, 1µg/mL leupeptin, 1µg/mL pepstatin A, pH 7.4). After centrifugation (5000 g, 4 °C, 5 min), supernatants containing 800 µg protein were then incubated with 2 µg of monoclonal SHP-1 or SHP-2 antibody (Santa Cruz) overnight at 4 °C on a rocking platform followed by immunoprecipitation with 30 µL GammaBind Plus Sepharose beads (GE Healthcare) for 3 additional hours. Immune complexes were washed 2 times in immunoprecipitation buffer and once in phosphatase assay buffer (25 mM Imidazole pH 7.2, 45 mM NaCl, 1 mM EDTA, 5 mm dithiothreitol, 1mg/mL BSA). Immune complexes were incubated in 100 µL phosphatase assay buffer with a final concentration of 20 mM p-nitrophenyl phosphate (P-NPP, Thermo Fisher Scientific) for 30 min at 37°C with shaking. Optical density of the supernatants was measured at 410 nm. After measurement of PTP activity, the proteins were recovered from the beads and subjected to SDS-PAGE gel for the western blotting analysis. The absorbance values obtained from PTP immune complexes were then normalized by the respective densitometry values of SHP-1 or SHP-2 immunoblots.

**FIGURE LEGEND:**

**Online FIGURE I:** Quantitative RT-PCR for angiotensin II AT1 and AT2 receptors (AT1R and AT2R, respectively). Total RNA was isolated from ventricles and kidneys of control and transgenic animals implanted with Ang II-releasing minipumps for 19 days and subjected to quantitative reverse-transcriptase polymerase chain reaction. Amplification curves were normalized for S16 ribosomal RNA. All signals are relative to Control, set at 1. Results are mean±SEM. N=6-8 animals per group.
Online Figure I