Cardiac Angiotensin-(1-7) in Ischemic Cardiomyopathy

David B. Averill, PhD; Yuichiro Ishiyama, MD; Mark C. Chappell, PhD; Carlos M. Ferrario, MD

Background—Accumulating evidence suggests that angiotensin-(1-7) (Ang-[1-7]) may play an important role in counteracting the pressor, proliferative, and profibrotic actions of angiotensin II in the heart. Thus, we evaluated whether Ang-(1-7) is expressed in the myocardium of normal rats and those in which myocardial infarction was produced 4 weeks beforehand.

Methods and Results—The left coronary artery in 10-week-old Lewis rats was either ligated (n=5) or exposed but not occluded in age-matched controls (sham; n=5). Left ventricular end-diastolic pressures were significantly elevated 4 weeks after myocardial infarction (25±1 versus 5±1 mm Hg for sham; P<0.001), whereas left ventricular systolic pressures were significantly reduced (ligated 86±4 versus sham 110±5 mm Hg; P<0.01). Hemodynamic effects of coronary artery ligation were accompanied by significant cardiac hypertrophy (heart weight to body weight: ligated 4.3±0.1 versus sham 2.9±0.1 mg/g; P<0.001). In both ligated and sham rats, Ang-(1-7) immunoreactivity was limited to cardiac myocytes and absent in interstitial cells and coronary vessels. Ang-(1-7) immunoreactivity was significantly augmented in ventricular tissue surrounding the infarct area in the heart of rats with myocardial infarction.

Conclusions—Development of heart failure subsequent to coronary artery ligation leads to increased expression of Ang-(1-7), which was restricted to myocytes. (Circulation. 2003;108:2141-2146.)

Key Words: heart failure ★ angiotensin ★ myocardial infarction ★ cardiomyopathy

Cardiac remodeling that occurs with afterload-induced cardiac hypertrophy or myocardial ischemia is associated with activation of the renin-angiotensin system, caused in part by increased expression of ACE in the heart.1–4 Augmented production of angiotensin (Ang) II in cardiac tissue contributes to the development of cardiac hypertrophy, apoptosis, and interstitial fibrosis.5 It is less clear, however, whether cardiac remodeling is associated with upregulation of renin and angiotensinogen in heart tissue.3

Emerging evidence shows that the vasopressor and proliferative actions of Ang II may be opposed by the production of Ang-(1-7) from either Ang I or Ang II.6,7 Local generation of Ang-(1-7) in the myocardium of dogs was demonstrated by Tipnis et al,8 and Santos et al9 found elevations of Ang-(1-7) in the myocardium of dogs.10,11 From The Hypertension and Vascular Disease Center, Wake Forest University School of Medicine, Winston-Salem, NC. Correspondence to David B. Averill, PhD, The Hypertension and Vascular Disease Center, Wake Forest University School of Medicine, Medical Center Blvd, Winston-Salem, NC 27157. E-mail daverill@wfubmc.edu © 2003 American Heart Association, Inc.

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ACE2 knockout mice [ACE2(-/-)] showed severe cardiac contractile dysfunction associated with ventricular dilation.14

The objective of the present study was to evaluate in rats whether Ang-(1-7) is present in cardiac tissue and to determine whether cardiac remodeling after myocardial infarction alters the expression of Ang-(1-7). Experiments were done in Lewis rats, in which coronary artery occlusion leads to a reproducible infarct size associated with cardiac failure.

Methods

Animal Procedures
Adult (10 weeks old; weight, 252±8 g) male Lewis rats (Charles River Laboratories, Wilmington, Mass) were anesthetized with ketamine (80 mg/kg IP) and xylazine (12 mg/kg IP), intubated, and placed on positive pressure ventilation. The thorax was entered via the left fourth intercostal space and the pericardium incised to expose the heart. In 5 rats, a 6-0 silk suture passed under the left main coronary artery was used to occlude this vessel (ligated group), whereas the left coronary artery was left untouched in 5 other rats (sham group). The thorax was closed and evacuated of fluid and air, and the animals were removed from the ventilator. Rats were housed individually for 4 weeks. After this time, animals were brought back to the laboratory, weighed, and anesthetized with halothane (1% to 2% in a mixture of 65% air and 35% oxygen). A plastic catheter (PE-10, Clay Adams) was inserted into the right carotid artery and advanced into the left ventricle. Left ventricular and arterial pressures and heart rate were determined with a computer-based data acquisition system (Biopac Instruments). After euthanasia, the heart was excised, the atria and aorta were removed, and the heart was advanced into the left ventricle. Left ventricular and arterial pressures and heart rate were determined with a computer-based data acquisition system (Biopac Instruments). After euthanasia, the heart was excised, the atria and aorta were removed, and the heart was

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weighed. A transverse midsection (2 to 3 mm in thickness) of the heart was obtained and placed immediately in 4% formalin. A portion of the transverse section was stained with picrosirius red to assess the area of the heart subjected to myocardial ischemia.

All procedures conformed to the Guidelines for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised in 1985). Research protocols were reviewed and approved by the Animal Care and Use Committee of Wake Forest University School of Medicine.

**Histology and Immunohistochemistry**

Cardiac tissue was left in 4% formalin for 48 hours before being transferred to 70% ethanol. Blocks of cardiac tissue were imbedded in paraffin; 5-μm sections were transferred to subbed slides and deparaffinized by sequential washes with xylene, 100% ethanol, 95% ethanol, 75% ethanol, and double-distilled water. Tissue sections were incubated with 3% hydrogen peroxide for 5 minutes, washed with PBS (pH 7.2), dried, and then incubated with 5% normal goat serum for 1 hour at room temperature. Sections were washed with PBS and incubated overnight at 4°C with an affinity-purified rabbit polyclonal antibody to Ang-(1-7) at 1:25 dilution of the antibody in 1% BSA. The Ang-(1-7) antibody was purified and characterized by us as described elsewhere. The next day, tissues were washed with PBS and incubated for 3 hours at 4°C with a biotinylated anti-rabbit antibody at a dilution of 1:400 in 1% BSA. Slides were rinsed with PBS and dehydrated in ethanol, 75% ethanol, and double-distilled water. Sections were dehydrated in ethanol (70% to 100%) and then Histoclear (National Diagnostics). Finally, they were mounted under coverslips with Histomount (National Diagnostics).

We employed several strategies to establish the selectivity of the affinity-purified antibody for Ang-(1-7). First, the specificity of staining obtained with the Ang-(1-7) antibody was assessed by preabsorption of the antibody with 10 μmol/L Ang-(1-7) (Bachem California). Second, incubation of biotinylated goat anti-rabbit IgG (Vector Laboratories) alone without primary antibody was used as a control to validate the staining procedure. Third, we performed immunoblots of membrane and soluble fractions of heart tissue for immunoreactivity to Ang-(1-7). For this procedure, frozen cardiac tissue was homogenized in PBS and centrifuged at 20,000g for 30 minutes at 4°C. The resultant supernatant and pellet fractions were diluted in SDS/β-ME buffer, boiled for 5 minutes, and applied to a 4% to 15% polyacrylamide gel. Proteins were transferred to PVDF membranes and blocked for 60 minutes in milk/0.05% Tween, and the membranes were incubated for 18 hours at 4°C with the affinity-purified Ang-(1-7) antibody (final dilution 1:25). Goat anti-rabbit IgG linked to horseradish peroxidase (1:2000) was incubated an additional 60 minutes, resolved with a Pierce Super Signal West Pico chemiluminescent kit (Pierce Biotechnology), and detected immunocytochemically by the avidin-biotin method (Vector Laboratories) and stained brown with 3,3’-diaminobenzidine (Sigma Chemical Co) in Tris buffered saline (pH 7.6 to 7.7). The intensity of staining obtained with the Ang-(1-7) antibody was assessed by Adobe Photoshop software (Zeiss). The digitized images at 20× and 100× magnifications were saved as JPEG files (1300×1030). The intensity of Ang-(1–7)-like immunoreactivity was quantified in Adobe Photoshop (version 5.5). To establish the type of cardiac cells staining for Ang-(1-7), we examined tissue sections stained with either picrosirius red alone or in combination with Ang-(1-7) immunohistochemistry. Picrosirius red selectively stains type I and III collagen fibers. Picrosirius red–stained tissue was examined under polarized light to more effectively identify collagen fibers in cardiac tissue.

**Analysis**

All measurements are expressed as mean±SEM computed from average results determined for tissue sections in each rat. Comparisons between rats with and without coronary artery occlusion were performed with a 2-tailed, unpaired Student t test (GraphPad Software). A probability value less than or equal to 0.05 was required for statistical significance.

**Results**

Hemodynamic and morphometric changes in rats subjected to either sham operation or coronary artery ligation are documented in Table 1. Ligation of the left coronary artery resulted in loss of functional myocardial mass, which averaged 44±1% of the left ventricle. Four weeks after coronary artery occlusion, rats exhibited bradycardia associated with significant reductions in left ventricular systolic pressures whereas left ventricular end-diastolic pressure (LVEDP) showed a 5-fold increase over values determined in sham-operated rats. In addition, left ventricular hypertrophy was present in rats 4 week after myocardial infarction (Table 1).

**Ang-(1–7) Staining in Ischemic Cardiomyopathy**

Ang-(1-7) immunoreactivity was found in myocytes of the right, left, and interventricular septum of both sham and coronary artery–ligated rats. Figure 1 illustrates staining for Ang-(1-7) in myocytes of the right ventricular free wall and interventricular septum of a Lewis rat subjected to coronary artery ligation. Similar staining was found in right and left ventricular tissue of rats without ligation of the left coronary artery. Because it was difficult to preserve good postmortem ventricular morphometry in all rats subjected to coronary ligation, analysis of Ang-(1-7)-immunoreactivity relied on examination of interventricular septal myocardium.

Selectivity of the affinity-purified antibody for Ang-(1-7) was demonstrated by the absence of reaction product when the antibody was preabsorbed with 10 μmol/L Ang-(1-7) (Figure 2). Incubation of the Ang-(1-7) antibody with either Ang II (10 μmol/L) or brain natriuretic peptides (BNP32 or BNP45 at 10 and 100 μmol/L, respectively) had no effect on immunoreactivity when tissue was reacted with preimmune serum and the secondary antibody. Immunoblot analysis of the supernatant fraction from heart homogenates with the Ang-(1-7) antibody revealed a single

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sham Group</th>
<th>Ligated Group</th>
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<tr>
<td>Heart rate, bpm</td>
<td>326±12</td>
<td>275±13</td>
<td>&lt;0.05</td>
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<tr>
<td>Left ventricular pressures</td>
<td></td>
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<tr>
<td>Systolic pressure, mm Hg</td>
<td>110±5</td>
<td>86±4</td>
<td>&lt;0.01</td>
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<tr>
<td>End-diastolic pressure, mm Hg</td>
<td>4.8±1.3</td>
<td>25.3±0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>373±12</td>
<td>356±9</td>
<td>NS</td>
</tr>
<tr>
<td>Heart weight/body weight ratio, mg/g</td>
<td>2.9±0.1</td>
<td>4.3±0.1</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean±SEM 4 weeks after either sham operation or coronary artery ligation.
Figure 1. Ang-(1-7) immunoreactivity in ventricular tissue of ischemic cardiomyopathic heart. A, Midsection of heart illustrating remodeling of left (LV) and right (RV) ventricles at 4 weeks after ligation of left coronary artery. Area outlined in free wall of left ventricle demarcates area of damage subsequent to occlusion of left coronary artery. Two boxes (I and II) indicate regions in each ventricular wall where higher-magnification photomicrographs were taken. These higher magnifications are illustrated in B, B, Ang-(1-7) immunoreactivity in cardiac myocytes of free wall of right ventricle (I) and ventricular septum (II) between left and right ventricle. Ang-(1-7) immunoreactivity is depicted as brown reaction product. Tissue was counterstained with hematoxylin to identify cell nuclei. Similar staining for Ang-(1-7) was observed in myocytes in 2 regions of heart.

Figure 2. A, Ang-(1-7) immunoreactivity in heart of Lewis rat subjected to ligation of left coronary artery; B, Ang-(1-7) immunoreactivity for same heart tissue when Ang-(1-7) antibody had been preabsorbed with 10 μmol/L Ang-(1-7).

1 band of 12 kDa that was distinct from the molecular weight of Ang-(1-7) (0.875 kDa). However, when cardiac tissue was prepared under nondenaturing conditions, we were unable to immunoprecipitate this protein with the Ang-(1-7) antibody. Figure 3 (A, B, C, and D) illustrates Ang-(1-7)-immunoreactivity in the interventricular septum of a sham-operated rat in which tissue was cut across either the longitudinal or transverse axis of cardiac myocytes. Ang-(1-7) staining was found throughout the cytoplasm of myocytes, whereas staining for the peptide was absent in interstitial cells and intramyocardial vessels.

Ang-(1-7) staining in hearts of Lewis rats subjected to ligation of the left coronary artery revealed Ang-(1-7) staining that differed from that seen in the sham-operated rats. First, the overall intensity of staining for Ang-(1-7) was visibly augmented within the interventricular myocardium (Figure 3, E, F, G, and H) and the myocardium immediately adjacent to the infarct area (also known as the penumbra). Quantification of the intensity of the brown reaction product for Ang-(1-7) immunoreactivity showed there were significant increases in Ang-(1-7) staining intensity in myocardium outside the region of ischemic damage (Table 2). The second important feature of Ang-(1-7) staining in the hearts of rat subjected to coronary artery ligation was the distinct absence of Ang-(1-7) immunoreactivity in fibroblasts and connective tissue cells in the region of the heart that had been perfused by the occluded left coronary artery, that is, the infarcted zone (Figure 4). Finally, coronary vessels showed no staining for Ang-(1-7) (Figure 3D). To further demonstrate that Ang-(1-7) was restricted to myocytes and not present in fibroblasts, we compared the Ang-(1-7) staining with adjacent tissue sections that were stained with picrosirius red. Figure 5 clearly shows that Ang-(1-7) immunoreactivity was localized exclusively in cells not stained with picrosirius red.

Two recent studies suggest that elevations in circulating Ang-(1-7) may offer some protection against the deterioration in cardiac function that occurs during the course of cardiac hypertrophy. On the other hand, Hirsch et al. posited that activation of the cardiac renin-angiotensin system is associated with ventricular dysfunction. To determine whether there may be a relationship between increased cardiac Ang-(1-7) and cardiac function, we examined the correlation between the intensity of Ang-(1-7) staining and LVEDP or maximum positive left ventricular dP/dt. Figure 6 shows that a significant (P<0.005) positive correlation existed between intensity of Ang-(1-7) staining and LVEDP, whereas a significant (P<0.05) inverse correlation was found between the intensity of Ang-(1-7) staining and the maximum positive left ventricular dP/dt.

Discussion

This study both extends and confirms the presence of Ang-(1-7) in the rat myocardium by revealing for the first time that immunoreactivity for Ang-(1-7) is restricted to cardiac myocytes. Furthermore, the intensity of Ang-(1-7) staining was increased in Lewis rats that developed ischemic cardiomyopathy 4 weeks after ligation of the left coronary artery. The increase in Ang-(1-7) staining was more intense in myocytes found in the penumbra of the zone of infarction.

The specificity and selectivity of the polyclonal antibody used for Ang-(1-7) immunohistochemistry was evaluated in several ways. In preliminary experiments, we found that affinity purification of rabbit antisera raised against Ang-(1-7) provided an antibody fraction that was highly selective for Ang-(1-7). In addition, there was no evidence for reaction product when immunohistochemistry was performed with preimmune serum used in place of the primary antibody for Ang-(1-7). Moreover, preabsorption of the primary antibody with 10 μmol/L Ang-(1-7) yielded no Ang-(1-7) staining. This control procedure was done on tissue obtained from the same tissue blocks of ligated and sham-ligated rats that had demonstrated Ang-(1-7) staining with a 1:25 dilution of the primary antibody. Immunoblots of heart tissue from Lewis rats revealed a single 12-kDa band in the supernatant fraction. However, absence of immunoprecipitation with the Ang-(1-7) antibody under
nondenaturing conditions most likely excludes this protein as being responsible for the positive immunocytochemical staining in the heart. In addition, the sensitivity of the quantification procedure used to assess the intensity of Ang-(1-7) staining was evaluated at 3 different dilutions (1:10, 1:25, and 1:50) of the primary antibody. This analysis revealed that a linear correlation existed between the intensity of staining and serial dilution of the antibody.

Zhang and coworkers demonstrated increases in Ang I and Ang II immunoreactivity in the myocardium of rats after myocardial infarction. In keeping with their findings, we examined the extent of Ang-(1-7) immunoreactivity in the right and left ventricular free walls and the interventricular septum of sham and ligated rats. In all cases, comparable staining for Ang-(1-7) immunoreactivity was restricted to myocytes of the right, left, and interventricular tissue of sham or ligated groups. These data suggest that increased Ang-(1-7) tissue staining in the hearts of ligated rats may reflect a response to the increased wall stress in both ventricles. A possible relation between increased wall stress and Ang-(1-7) staining is supported by the finding of more pronounced staining for this peptide in myocytes immediately adjacent to regions of ischemic damage. In addition, increased accumulation of Ang-(1-7) immunoreactivity correlated directly with increased LVEDP and inversely with decreased maximum left ventricular dP/dt. These data suggest that the expression of Ang-(1-7) in cardiac tissue is linked to changes in cardiac contractility.

**TABLE 2. Differential Effects of Coronary Artery Ligation on Immunoreactive Ang-(1–7) Staining**

<table>
<thead>
<tr>
<th>Left Ventricular Section</th>
<th>×20 Magnification</th>
<th>×100 Magnification</th>
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<tbody>
<tr>
<td></td>
<td>Sham Group (n=5)</td>
<td>Ligated Group (n=5)</td>
</tr>
<tr>
<td>Longitudinal axis</td>
<td>88.9±10.5</td>
<td>106.0±10.5</td>
</tr>
<tr>
<td></td>
<td>87.7±6.4</td>
<td>107.1±8.3</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Transverse axis</td>
<td>85.9±4.7</td>
<td>113.4±4.3</td>
</tr>
<tr>
<td></td>
<td>92.6±13.2</td>
<td>113.7±13.3</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are mean±SEM for intensity of staining as determined by analysis in Photoshop (version 5.5). The range for intensity was from 0 (lightest) to 255 (darkest). On average, tissue sections stained with the Ang-(1–7) antibody preabsorbed with 10 mmol/L Ang-(1–7) had intensities ranging from 45.6 to 74.0.
Zhang et al.\textsuperscript{22} observed Ang I and Ang II staining both in cardiac myocytes and in interstitial cells of the heart. Similar findings for Ang II immunoreactivity have been reported in patients with heart failure (New York Heart Association classes I through IV).\textsuperscript{2} In contrast, we found that Ang-(1-7) staining was restricted to cardiac myocytes, a finding that is in keeping with compartmentalization of either products or pathways leading to the diverse formation of angiotensin peptides. Cardiac remodeling that occurs after myocardial ischemia is associated with infiltration of fibroblasts and deposition of collagen fibers between myocytes and in areas of ischemic damage. By staining collagen fibers with picrosirius red, we were able to determine that fibroblasts and other collagen-containing cells were devoid of Ang-(1-7) staining.

In previous experiments, we showed release of Ang-(1-7) in the coronary sinus of dogs after acute myocardial infarction.\textsuperscript{31} whereas in the same species, infusion of Ang I was accompanied by a significant recovery of Ang-(1-7) from the myocardial interstitium.\textsuperscript{8} There is evidence that cardiac cells may take up angiotensinogen, renin, or even Ang I from the plasma compartment.\textsuperscript{3} This may be important, because there appears to be low expression of renin and angiotensinogen in heart tissue.\textsuperscript{3,24} The exact pathway responsible for production of angiotensin peptides in cardiac tissue is a developing story since Nguyen et al.\textsuperscript{25} recently reported the cloning of a membrane-bound receptor for renin in humans. Because the renin receptor was localized to smooth muscle cells of coronary blood vessels, uptake of renin from the circulation into heart tissue may be part of the pathway that contributes to production of angiotensin peptides. Finally, the identification of cardiac ACE2, an enzyme that exhibits high catalytic efficiency for the conversion of Ang II to Ang-(1-7), and the presence of significant cardiac abnormalities after ACE2 gene deletion in mice further support the existence of multiple angiotensin-processing pathways within the heart.\textsuperscript{14}

In summary, these studies add anatomic evidence for a potential role of Ang-(1-7) in the regulation of cardiac myocyte function and demonstrate that loss of myocardial mass is associated with increased Ang-(1-7) expression in tissue surrounding the infarcted area. The demonstration by Loo et al.\textsuperscript{10} that Ang-(1-7) attenuates the development of heart failure in rats with myocardial infarction provides functional evidence for the data presented here.

Acknowledgments

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References


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