Increased Angiotensin-(1-7)–Forming Activity in Failing Human Heart Ventricles

Evidence for Upregulation of the Angiotensin-Converting Enzyme Homologue ACE2

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Background—The formation of angiotensin-(1-7) from either angiotensin (Ang) I or Ang II in failing human hearts is not well understood.

Methods and Results—Angiotensinase activity in left and right ventricular membranes from 14 idiopathic dilated cardiomyopathy (IDC), 8 primary pulmonary hypertension (PPH), and 13 nonfailing human hearts was measured with either 125I-Ang I or 125I-Ang II as substrate. Ang-(1-7)–forming activity from 125I-Ang I was inhibited by thiorphan. With 125I-Ang II as substrate, Ang-(1-7) formation was inhibited by the ACE2-specific inhibitor C16. Western blotting with an anti-ACE2 antibody confirmed the presence of ACE2. Angiotensinase activity with 125I-Ang I as substrate was increased in failing IDC left ventricles (LVs) compared with nonfailing LVs (P<0.001). Ang-(1-7)–forming activity with 125I-Ang II as substrate was increased in both failing LVs and right ventricles (RVs) of IDC hearts and only in failing RVs of PPH hearts (PPH LV, 51.12±5.25; PPH RV, 89.97±11.21; IDC LV, 139.7±21.96; and IDC RV, 192.7±5.43; NF LV, 32.89±5.38; NF RV 40.49±10.66 fmol/min per milligram (P<0.05 PPH RV versus PPH LV; P<0.05 PPH RV versus NF RV; P<0.001 IDC LV versus NF LV; P<0.001 IDC RV versus NF RV).

Conclusions—Ang-(1-7)–forming activity from both Ang I and Ang II was increased in failing human heart ventricles but was mediated by at least two different angiotensinases. The first, which demonstrated substrate preference for Ang I, was neutral endopeptidase (NEP)-like. The second was ACE2, as demonstrated by Western blotting and inhibition of activity with C16. (Circulation. 2003;108:1707-1712.)

Key Words: angiotensin enzymes cardiomyopathy

Angiotensin-converting enzyme (ACE) inhibitor therapy has been shown to improve survival and cardiac function in patients with heart failure. Presumably, a significant benefit of this therapy results from a reduction of angiotensin (Ang) II concentration in the heart. However, the regulation of Ang II metabolism in the failing human heart is not well understood. Recently, the set of enzymes that hydrolyze Ang I and Ang II directly to the smaller angiotensin peptide Ang-(1-7) have received increased attention. Enzymes that have been shown to hydrolyze Ang I and/or II to Ang-(1-7) include angiotensinase C, prolylendopeptidases, neutral endopeptidase, endothelin-converting enzyme, and the recently described homologue of ACE, ACE2 (or ACEH). ACE2 has been shown to hydrolyze Ang I to Ang-(1-9) and the recently described homologue of ACE, ACE2 (or ACEH). ACE2 has been shown to hydrolyze Ang I to Ang-(1-9) and Ang II to Ang-(1-7). It has been proposed that Ang-(1-7) counteracts the effects of Ang II. There is a growing body of evidence that Ang-(1-7) exerts counterregulatory effects through a specific pathway involving a novel Ang-(1-7) receptor and possibly cross-talk with the bradykinin pathway. The ACE2-knockout mouse model resulted in a heart failure phenotype, a finding that has reinvigorated interest in the regulation of angiotensin metabolism in human heart failure.

In the present study, we report that Ang-(1-7)–forming activity is increased in failing human heart ventricles and that the profile of Ang-(1-7) formation from Ang I is different than that from Ang II. We identify the enzyme responsible for increased Ang-(1-7) formation from Ang I in failing human heart ventricles as ACE2.

Methods

Materials

[125I] Tyr4-angiotensin II and [125I] Tyr4-angiotensin I were purchased from Perkin-Elmer Life Sciences. The Poros HQ anion exchange...
column was obtained from Applied Biosystems. Ethylenediaminetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), p-chloromercuripenylsulfonate (p-CMPS), bestatin, thiorphan, potato carboxypeptidase A inhibitor, phosphoramidon, and phenylmethylsulfonylfluoride (PMSF) were purchased from Sigma Chemical Co. Z-pro-prolinal was obtained from Bachem. Ramiprilat was a gift from Hoechst Marion Roussel, Inc. The ACE2-specific inhibitor compound 16 (C16) was a kind gift of Dr Natalie Dales,23 and the ACE2-specific polyclonal antibody K70417K directed against amino acids 489-508 was a kind gift of Dr Susan Acton (both of Millennium Pharmaceuticals, Cambridge, Mass). All other chemicals were of reagent grade from Sigma. Custom radioiodination of Ang III, IV, 1-5, 1-7, and 1-9 were performed by the radioiodination peptide service at Washington State University, Pullman, Wash.

**Clinical Characteristics of Research Subjects**

Hearts were obtained from 14 patients with end-stage idiopathic dilated cardiomyopathy (IDC), 8 patients with primary pulmonary hypertension (PPH), and 13 organ donors. The IDC hearts were obtained at the time of heart transplantation, and the PPH hearts were obtained at the time of combined heart/lung transplantation. Hearts from subjects with PPH had selective failure of the right ventricle, with preserved left ventricular function. Characteristics of the PPH hearts were as previously described.22 The hearts from the organ donors had normal left ventricular systolic function. Nonfailing (NF) controls, as previously described,22 used: EDTA, Z-pro-prolinal: 200 μmol/L, thiorphan: 10 μmol/L; carboxypeptidase A inhibitor: 10 μmol/L; enalaprilat: 10 μmol/L; ramiprilat, 10 μmol/L; phosphoramidon: 300 μmol/L; C16: 10 μmol/L.

**Angiotensinase Assay**

Angiotensinase assays were performed under conditions similar to a previously described Ang II-forming activity assay.22,23 The reaction mixture (200 μL total volume) contained 10 to 15 μg protein (25 μL), 200 fmol of 125I-Ang I or II, and assay buffer (175 μL). Incubation was performed for 20 minutes at 37°C. The reaction was quenched by addition of 0.085% H₃PO₄ (600 μL) plus methanol (430 μL). The HPLC separation was as previously described,22,23 with slight modifications. Mobile phase A was 0.085% H₃PO₄ and B was 100% methanol. The Nucleosil C₁₈ column operated at 45°C. A series of mobile phase B gradients were applied (20% to 24% B in 4 minutes, 24% to 37.5% B in 17 minutes, 37.5% to 45% B in 10 minutes, 45% to 80% B in 8 minutes, and 80% for 5 minutes). Retention times were as follows: 125I-Ang I, 36.8; 125I-Ang II, 27.6; 125I-Ang III, 24.7; 125I-Ang IV, 30.5; 125I-Ang-(1-5), 23.2; 125I-Ang-(1-7), 16; and 125I-Ang-(1-9), 19.7 minutes.

**Chromatographic Separation of Angiotensinases**

Solubilized/dialyzed samples were fractionated with a Poros HQ column. After sample application, the column was washed with equilibration buffer (20 mmol/L Tris-HCl, 0.02% (vol/vol) Trition X-100, pH7.5) followed by a linear gradient of 0 to 250 mmol/L NaCl and step to 800 mmol/L NaCl. The column effluent was collected in 1-mL fractions. Angiotensinase activity assays were performed for each fraction with either 125I-Ang I or 125I-Ang II as the substrate. For inhibition studies, the following concentrations were used: EDTA, Z-pro-prolinal: 20 μmol/L; p-CMPS: 20 μmol/L; PMSE: 100 μmol/L; thiorphan: 10 μmol/L; carboxypeptidase A inhibitor: 10 μmol/L; enalaprilat: 10 μmol/L; ramiprilat, 10 μmol/L; phosphoramidon: 300 μmol/L; C16: 10 μmol/L.

**Measurement of ACE Protein and Angiotensin Receptor Concentrations**

ACE protein, total angiotensin receptor, and angiotensin receptor subtype 1 (AT1) and 2 (AT2) concentrations were measured as previously described.22,24 ACE protein was quantified in 11 IDC left ventricles (LVs) and 9 NF LVs in which Ang-(1-7) formation was also measured. Angiotensin receptor density was measured in 7 PPH right ventricles (RVs) in which Ang-(1-7)-forming activity was also determined.

**Western Blot Analysis**

Four micrograms of protein were added to sample buffer (×2, 4% SDS, 100 mmol/L tris-CI, pH 6.8, 20% glycerol, 0.2% bromphenol blue, 200 mmol/L dithiothreitol) and boiled for 10 minutes. SDS-PAGE was performed with an 8% to 16% gradient gel and transferred to nitrocellulose (BioRad Electrophoresis/Transfer Apparatus). Nitrocellulose was blocked overnight in 5% milk in TBS containing 0.2% Tween-20 (TBST) at room temperature. The membrane was incubated in ACE2 polyclonal antibody diluted 1:5000 in 1% BSA/TBST for 1.5 hours, washed (×3 ×15 minutes TBST), and incubated in donkey-anti-rabbit HRP antibody (1:1500, Amersham) for 1 hour and washed (3×15 minutes TBST). ECL (Amersham) and blue film (Empire Imaging) were used for detection of ACE2. The ACE2 band was visualized at an apparent molecular weight of 130 kDa.

**Statistical Analysis**

Comparisons between groups were performed with ANOVA followed by the Newman-Keuls multiple comparison test. Correlation analysis was performed to determine if there was a relation between angiotensinase activity, ACE protein, angiotensin receptor density, Ang II-
forming activity, and prior treatment with ACE inhibitors, diuretics, digoxin, or inotropes. Statistical significance was set at the $P < 0.05$ level. Unless stated otherwise, values are given as mean $\pm$ SEM.

**Results**

Angiotensin elution profiles for the solubilized and nonsolubilized membrane fractions with either $^{125}$I-Ang I or $^{125}$I-Ang II substrates are shown in Figure 1. With $^{125}$I-Ang I as substrate, both Ang II and Ang-(1-7)–forming activities were detected in the solubilized fraction; however, no Ang-(1-9)–forming activity was found in this fraction. Ang-(1-9)–forming activity was restricted to the nonsolubilized pellet. With $^{125}$I-Ang II as substrate, Ang-(1-7)–forming activity was restricted to the solubilized membrane fraction.

Ang-(1-7)–forming activity with $^{125}$I-Ang I as substrate was significantly increased in IDC left ventricles compared with NF LVs: NF LV, 37.86 $\pm$ 1.96; IDC LV, 71.3 $\pm$ 7.29 fmol/min per milligram ($P < 0.001$, Figure 2A). Ang-(1-7)–forming activity was not significantly increased in PPH LVs, PPH RVs, or IDC RVs compared with donor heart nonfailing LVs or RVs: PPH LV, $P < 0.05$ vs NF LV; $P < 0.001$ vs NF RV and PPH LV. $P < 0.001$ vs NF RV.
56.10±3.96; PPH RV, 60.33±5.25; IDC RV, 48.94±5.78; NF RV, 43.69±6.17 fmol/min per milligram; 125I-Ang-(1-7)–forming activity was not significantly different between ACEI and NF hearts (PPH LV, 51.12±5.25; PPH RV, 89.97±11.21; IDC LV, 139.7±21.96; and IDC RV, 192.7±5.43; NF LV, 32.89±5.38; NF RV 40.49±10.66 fmol/min per milligram; P<0.05 PPH RV versus NF RV; P<0.001 IDC LV versus NF LV; P<0.001 IDC RV versus NF RV). Ang-(1-7) formation from 125I-Ang II substrate was higher in the PPH RVs compared with PPH LVs (P<0.05) and the NF RVs (P<0.01, Figure 2B). Angiotensinase activity was not significantly different in the IDC subjects treated with ACE inhibitors (ACEI+) compared with those who were not receiving ACE inhibitors before transplantation (ACEI−). With 125I-Ang I substrate, IDC LVs: ACEI+, 69.53±9.05, versus ACEI−, 77.88±9.22 fmol/min per milligram 125I-Ang-(1-7) generated; IDC RVs: ACEI+, 45.71±4.63 versus ACEI−, 52.97±7.78 fmol/min per milligram (P=NS.) With 125I-Ang II substrate, IDC LVs: ACEI+, 148.2±33.51, versus ACEI− 123.3±3.21 fmol/min per milligram; IDC RVs: ACEI+, 198.2±1.75, versus ACEI−, 192.7±9.57 fmol/min per milligram (P=NS.) It has been reported that ACE can metabolize Ang-(1-7) to the smaller angiotensin fragment Ang-(1-5).26 Therefore, we performed angiotensinase assays with 125I-Ang II as substrate in the presence of the ACE inhibitor enalaprilat. However, no effect on Ang-(1-7)–forming activity was observed (data not shown).

To determine which angiotensinases might be responsible for the major portion of Ang-(1-7)–forming activity in these failing human heart preparations, we performed incubations of IDLV membrane preparations with either 125I-Ang I or II and various combinations of known angiotensinase inhibitors. With 125I-Ang I as substrate, there was a significant reduction in 125I-Ang-(1-7) formation in the presence of thiorphan but no significant effect of the selective ACE inhibitor enalaprilat (vehicle, 80.58±7.39; enalaprilat, 65.95±6.41; thiorphan, 24.82±7.42 fmol/min per milligram; P<0.001, n=11, Figure 3A). At the concentration used, both enalaprilat and thiorphan inhibited ACE activity and consequently decreased 125I-Ang II formation from 125I-Ang I (Figure 3B). These data indicate that the effect of thiorphan on Ang-(1-7) formation from Ang I was the result of its inhibition of neutral endopeptidase (NEP).

In another set of experiments, we examined the effect of the following inhibitors on Ang-(1-7)–forming activity when 125I-Ang II was used as the substrate: an inhibitor cocktail containing Z-pro-prolinal (ZP), p-CMPS, thiorphan, and carboxypeptidase A inhibitor (ZPTC), ZP alone, thiorphan alone, and the ACE2-specific inhibitor C16. The results of incubations performed with four IDC LV preparations are shown in Figure 4. In this series, angiotensinase activity was 113.9±0.5, 115.2±10.79, 119.6±2.8, and 116.7±8.8 fmol/min per milligram for vehicle, ZPTC, ZP, and thiorphan, respectively. C16 (at a concentration of only 10 nmol/L) reduced activity to 0.1±0.05 fmol/min per milligram (P<0.001). Separation of Ang-(1-7)–forming angiotensinase with substrate preference for Ang I from the Ang-(1-7)–forming angiotensinase with substrate preference for Ang II was achieved with a Poros HQ column. The separation and activity assays were performed with solubilized fractions from a failing IDC LV and a nonfailing LV. The activity of both angiotensinases was higher in the failing compared with nonfailing heart (Figure 5). The angiotensinase with substrate preference for Ang I eluted from the Poros HQ column was inhibited by pCMPS and thiorphan but not by Z-pro-prolinal, suggesting that this angiotensinase was NEP-like. The angiotensinase with substrate preference for Ang II eluted from the Poros HQ column was inhibited by C16. Western blot analysis with the anti-ACE2 antibody confirmed the presence of ACE 2 (Figure 5, inset).

There was a direct correlation between ACE protein concentration (ACE B max) and 125I-Ang II formation from 125I-Ang I substrate in 11 IDC and 9 NF LVs (r=0.23, P<0.0001). In the same set of hearts, a direct correlation between 125I-Ang-(1-7) formation and ACE B max was found (r=0.48, P<0.0001). However, the correlation between ACE B max and the sum of 125I-Ang-(1-7) + 125I-Ang II formation (r=0.44, P<0.0001; 125I-Ang II substrate) was higher than the correlation of ACE B max and 125I-Ang II formation alone. In addition, a direct correlation between 125I-Ang-(1-7)–forming activity and 125I-Ang II–forming activity was found (r=0.35, P<0.0001).

![Figure 3](https://circ.ahajournals.org/doi/10.1161/CIRCULATIONAHA.103.461041.Figure3)

**Figure 3.** A, Inhibition profile of 125I-Ang-(1-7) formation in failing human heart LV with Ang I as substrate. (*P<0.01 vs vehicle or enalaprilat [Enal]). B, Inhibition profile of 125I-Ang II–forming activity with 125I-Ang I substrate. (*P<0.01 vs vehicle.) Incubation in the presence of the ACE-inhibitor enalaprilat, which suppressed 125I-Ang II formation, did not decrease 125I-Ang-(1-7) formation from 125I-Ang I
Total and subtype (1 and 2) angiotensin receptor density was measured in the PPH hearts. There was a direct correlation between total angiotensin receptor density in the PPH RVs and $^{125}\text{I}-\text{Ang-(1-7)}$ formation with $^{125}\text{I}-\text{Ang II}$ substrate ($r=0.78$, $P<0.01$). This correlation was explained by a direct correlation with the AT2 receptor concentration ($r=0.76$, $P=0.01$; Figure 6). There was no significant relation found between Ang-(1-7) formation and the angiotensin subtype 1 receptor concentration.

Correlation analysis did not show a significant relation between angiotensinase activity and prior use of ACE inhibitors, digoxin, furosemide, or inotropes.

**Discussion**

In this study, we report for the first time increased Ang-(1-7)-forming activity in failing human heart ventricles. The conversion of $^{125}\text{I}-\text{Ang I}$ to $^{125}\text{I}-\text{Ang-(1-7)}$ was increased in IDC LVs and appeared to be NEP-like. The conversion of Ang II to Ang-(1-7) was selectively increased in the failing human heart ventricles from subjects with PPH or IDC and was mediated by ACE2.

We have previously demonstrated differential regulation of ACE and AT1 receptor protein concentration in failing human heart ventricles. In that study, there was a direct correlation between ACE protein concentration and $^{125}\text{I}-\text{Ang I}$ formation with $^{125}\text{I}-\text{Ang II}$ substrate ($r=0.76$, $P=0.01$; Figure 6). There was no significant relation found between Ang-(1-7) formation and the angiotensin subtype 1 receptor concentration.

Correlation analysis did not show a significant relation between angiotensinase activity and prior use of ACE inhibitors, digoxin, furosemide, or inotropes.

**Figure 5.** Elution profile of $^{125}\text{I}-\text{Ang-(1-7)}$-forming activity in failing and nonfailing human heart LV from a Poros HQ column. A, $^{125}\text{I}-\text{Ang-(1-7)}$-forming activity in each eluted fraction with $^{125}\text{I}-\text{Ang I}$ as substrate. B, $^{125}\text{I}-\text{Ang-(1-7)}$-forming activity with $^{125}\text{I}-\text{Ang II}$ as substrate. Fraction 19 showed greater activity with the substrate $^{125}\text{I}-\text{Ang I}$ and fraction 23 showed greater activity with substrate $^{125}\text{I}-\text{Ang II}$. In addition, with $^{125}\text{I}-\text{Ang I}$ as substrate, peak angiotensinase activity in fraction 19 was higher in the IDC preparation, whereas peak angiotensinase activity with $^{125}\text{I}-\text{Ang II}$ was higher in fraction 23 of the IDC compared with the NF preparation. Inset: Western blot with anti-ACE2 antibody demonstrated presence of ACE2 in the fractions with peak angiotensinase activity ($^{125}\text{I}-\text{Ang II}$ substrate.)

**Figure 6.** In PPH RVs ($n=7$), a significant correlation between AT2 receptor concentration and Ang-(1-7)-forming activity ($^{125}\text{I}-\text{Ang II}$ substrate) was found ($r=0.76$, $P=0.01$). There was no significant relation between the AT1 receptor subtype concentration and Ang-(1-7)-forming activity.
a direct relation between Ang-(1-7)-forming activity and AT2 receptor density (Figure 6). AT2 receptors appear to be regulated during development and to mediate antiproliferative and apoptotic signaling in some studies. However, a direct relation between Ang-(1-7) formation and AT2 receptor density is unlikely because Ang-(1-7) is not a ligand for this receptor. A growing body of evidence suggests that there is a distinct Ang-(1-7) receptor. Our data support the hypothesis that the correlation between Ang-(1-7) formation and AT2 receptor density in PPH RVs could arise as a result of cross-talk between the AT2 receptor and the putative Ang-(1-7) receptor.

The novel homologue of ACE, termed ACE2 or ACEH, was recently discovered by high-throughput screening of a human heart cDNA library. Initially, ACE2 was described as hydrolyzing Ang I to Ang-(1-9). However, subsequently, it has been demonstrated that ACE2 has a much lower Km for the hydrolysis of Ang II to Ang-(1-7). In our study, Ang-(1-9)-forming activity was restricted to the nonsolubilized pellet fraction. However, in this nonsolubilized fraction, no Ang-(1-7) formation from Ang II could be detected. This finding suggests that a carboxypeptidase other than ACE2 is primarily responsible for Ang-(1-9) formation in this fraction. In the solubilized fraction, there was evidence for NEP-mediated Ang-(1-7) formation from Ang I. However, Ang-(1-7) formation from Ang II was inhibited only by the ACE2-specific inhibitor C16. Indeed, the ACE2-specific inhibitor virtually eliminated all Ang-(1-7) formation from Ang II at a concentration of only 10 nmol/L. Finally, Western blot analysis with an anti-ACE2 antibody confirmed the presence of ACE2 in the membrane fraction from failing human heart ventricles.

Limitations
On homogenization of tissue, many enzymes are released that may never encounter Ang I or II in vivo. We attempted to focus our attention on membrane-bound angiotensinasines by using a solubilized membrane preparation of human heart ventricles because membrane-bound angiotensinasines are more likely to be physiologically relevant.

Conclusions
Ang-(1-7)-forming activity from both Ang I and Ang II was increased in failing human heart ventricles but was mediated by at least two different angiotensinasines. The first, which demonstrated substrate preference for Ang I, was NEP-like. The second was identified as ACE2. Increased Ang-(1-7) formation could serve as a counterregulatory, cardioprotective function in heart failure. Strategies to further augment Ang-(1-7)-forming activity and/or signaling through the Ang-(1-7) receptor warrant further investigation as a new approach to heart failure therapeutics.

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References
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