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
column was obtained from Applied Biosystems. Ethylenediaminetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethyl)ether)-N,N'-N'-tetraacetic acid (EGTA), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), p-chloromercuriphenylsulfonate (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, 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 using the NF hearts for heart transplantation were related to ABO blood type or donor/recipient size mismatch. A description of patient characteristics and cardiac hemodynamics is shown in the Table. Eleven patients with IDC were receiving ACE inhibitors; 6 were receiving digoxin; 9 were receiving high-dose diuretics; 5 were receiving dobutamine; 2 were receiving dopamine; and 1 was receiving enoximone at the time of explantation, as previously described. For the Ang-(1-7)–forming activity assay as well as for Western blot analysis, the angiotensinases 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; PMSF: 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.

**Chromatographic Separation of Angiotensinases**

Solubilized/dialed 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) Triton X-100, pH 7.5) 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; PMSF: 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. 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-Cl, 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 nonsolubilized pellet. With $^{125}$I-Ang II as substrate, Ang-(1-7)–forming activity was restricted to the nonsolubilized pellet. With $^{125}$I-Ang II as substrate, Ang-(1-7)–forming activity was restricted to the nonsolubilized pellet.

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 with $^{125}$I-Ang II substrate was increased in IDC LVs compared with NF LVs, IDC RVs compared with the NF RVs, PPH RV compared with NF RV, and PPH RV compared with PPH LV. In addition, angiotensinase activity with $^{125}$I-Ang II substrate was higher in IDC RVs compared with IDC LVs ($P < 0.001$).
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 IDC LVs with 125I-Ang I or II and various substrate in the presence of the ACE inhibitor enalaprilat. However, no effect on Ang-(1-7)-forming activity was observed with 125I-Ang II as substrate. The ACE2-specific inhibitor C16 virtually prevented 125I-Ang-(1-7) formation in failing LVs (Figure 3A). At the concentration of only 10 nmol/L it 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 I 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 3A. 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 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 $^{125}$I-Ang-(1-7) formation with $^{125}$I-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 $^{125}$I-Ang I to $^{125}$I-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.$^{22}$ In that study, there was a direct correlation between ACE protein concentration and $^{125}$I-Ang II–forming activity. Interestingly, whereas ACE protein was increased in both the nonfailing PPH LV and failing PPH RV, there was selective downregulation of the AT1 receptor only in the failing RV.$^{22}$ Our data herein show selective upregulation of ACE2 activity in the failing PPH RV but not the PPH LV. An even more dramatic increase in ACE2 activity was found in the IDC LVs and RVs.

We also examined the relation between Ang-(1-7)-forming activity, Ang II–forming activity, and ACE protein in IDC and NF LVs. The correlation between ACE $B_{\text{max}}$ and combined Ang-(1-7) + Ang II–forming activity was higher than the correlation of ACE $B_{\text{max}}$ and Ang II formation alone. Furthermore, there was a strong correlation between Ang-(1-7)–forming activity and Ang II–forming activity. These data indicate that as Ang II was formed, it was efficiently hydrolyzed to Ang-(1-7) and that Ang-(1-7) formation was dependent on substrate availability of Ang II.

The relation between Ang-(1-7)–forming activity and angiotensin receptor density in the PPH hearts was determined. A direct correlation between Ang-(1-7) formation and total angiotensin receptor membrane concentration in the PPH RVs was found; furthermore, this correlation was the result of
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 mmol/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 angiotensinases by using a solubilized membrane preparation of human heart ventricles because membrane-bound angiotensinases 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 angiotensinases. 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 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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