Heart Failure

Detection of Soluble Angiotensin-Converting Enzyme 2 in Heart Failure

Insights Into the Endogenous Counter-Regulatory Pathway of the Renin-Angiotensin-Aldosterone System

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Objectives	We sought to determine whether circulating soluble angiotensin-converting enzyme 2 (sACE2) is increased in the plasma of patients with heart failure (HF).
Background	Angiotensin-converting enzyme 2 (ACE2) is an integral membrane protein that antagonizes the actions of angiotensin II and prevents the development of HF in animal models. However, because of the need for invasive cardiac tissue sampling, little is known about whether ACE2 is involved in the pathophysiology of HF in humans.
Methods	We developed a sensitive and specific assay to measure sACE2 activity in human plasma and screened a heterogeneous group of patients suspected of having clinical HF.
Results	Increasing sACE2 plasma activity strongly correlated with a clinical diagnosis of HF ($p = 0.0002$), worsening left ventricular ejection fraction ($p < 0.0001$), and increasing B-type natriuretic peptide levels ($p < 0.0001$). Similar to B-type natriuretic peptide, sACE2 activity reflected the severity of HF, with increasing levels associated with worsening New York Heart Association functional class ($p < 0.0001$). These associations were independent of other disease states and medication use. We found that sACE2 activity was increased in patients with both ischemic and nonischemic cardiomyopathies and also in patients with clinical HF but a preserved left ventricular ejection fraction.
Conclusions	Soluble ACE2 activity is increased in patients with HF and correlates with disease severity, suggesting that a cardioprotective arm of the renin-angiotensin-aldosterone system is active in HF. (J Am Coll Cardiol 2008;52: 750–4) © 2008 by the American College of Cardiology Foundation

Although the pathophysiology of heart failure (HF) is complex, an important final common pathway involves the activation of the renin-angiotensin-aldosterone system (RAAS). Angiotensin-converting enzyme (ACE) mediates

the cleavage of angiotensin (Ang) I into Ang II, a peptide that acts downstream on specific angiotensin type I (AT-1) receptors, which together are all important targets for modern HF therapeutics. Angiotensin-converting enzyme 2

(ACE2) is a recently discovered homolog and, in contrast to other members of RAAS, ACE2 is seemingly protective in HF (1). It is an integral membrane carboxypeptidase that removes the terminal amino-acid from Ang II, resulting in the production of a biologically active peptide, Ang(1-7), that antagonizes the effects of Ang II by activating the Mas receptor (2,3). Mice that are deficient in ACE2 have increased Ang II levels and a severe cardiac contractility defect that is reversed by the genetic deletion of ACE (4). Intracardiac overexpression of ACE2 prevents Ang II-induced hypertension and cardiac fibrosis, implicating a direct in vivo cardioprotective role for ACE2, in addition to suggesting possible therapeutic utility (5).

ACE2 gene expression is increased in tissue samples from patients with left ventricular dysfunction (6). Traditionally, ACE2 has been thought to be tissue bound and, as a result, the need for invasive cardiac tissue sampling has limited additional clinical studies. Herein, we examine the feasibility of detecting

soluble angiotensin-converting enzyme 2 (sACE2) activity in human plasma. We then hypothesized that the endogenous regulatory arm of the RAAS, as measured by sACE2, would be increased in patients with HF.

Methods

ACE2 enzymatic assay. The ACE2-specific quenched fluorescent substrate protocol was performed as previously described with modifications (7). Human plasma (Bioreclamation, Hicksville, New York), and patients' plasma samples were diluted in a ratio of 30:70 with enzyme buffer (1 mol/l NaCl, 75 mmol/l Tris-HCl, 5 mmol/l $ZnCl_2$, pH 6.5) in the presence of protease inhibitors, including captopril 10 μmol/l, amastatin 5 μmol/l, bestatin 10 μmol/l (all from Sigma Chemical Co., St. Louis, Missouri), and Z-Pro-prolinal 10 μmol/l (Biomol International, L.P., Plymouth Meeting, Pennsylvania). Samples were incubated with quenched fluorescent substrate diluted in enzyme buffer
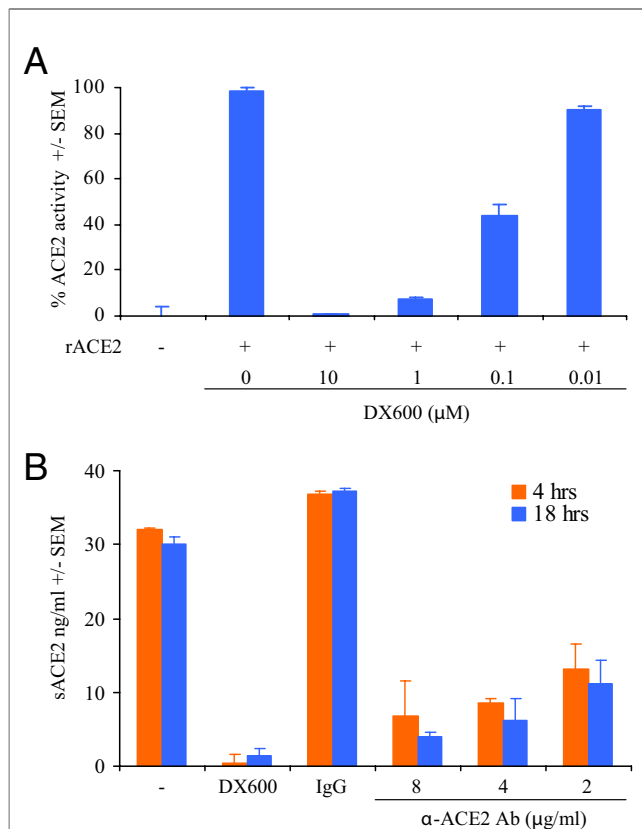

Figure 1   Plasma ACE2 Enzymatic Assay Specificity

(A) rACE2 (25 ng/ml) was mixed with 30% commercial human plasma in the presence of different concentrations of the specific ACE2 inhibitor DX600, and ACE2 activity was determined after incubation for 18 h. Data are expressed as the percentage of ACE2 activity of rACE2. **(B)** Thirty percent human plasma (patient sample) was pre-incubated with either DX600 (1 μmol/l), anti-human ACE2 polyclonal antibody, or isotype-matched control antibodies for 30 min and then ACE2 activity was determined at 4 and 18 h. One of 3 representative experiments is shown. ACE = angiotensin-converting enzyme; IgG = immunoglobulin G; rACE2 = recombinant angiotensin-converting enzyme 2 activity; SACE = soluble angiotensin-converting enzyme.

(final concentration 50 μmol/l, 100 μl) at 37°C. Maximal fluorescence (SpectraMax GeminiXS, Molecular Devices, Wokingham, United Kingdom) was determined by experimentation (λex = 324 nm, λem = 430 nm, using a 420-nm cutoff filter). Specificity was determined by pre-incubating plasma for 30 min with either the specific human ACE2 inhibitor DX600 (Phoenix Pharmaceuticals, Burlingame, California) (8), anti-ACE2 polyclonal antibodies, or control polyclonal antibodies (R&D Systems, Inc., Minneapolis, Minnesota). Each patient's plasma sACE2 activity was determined at 18 h, with 1 h as the baseline. Values were normalized to a recombinant ACE2 standard curve (R&D Systems). Intra-assay variability was 6.1 $\pm$ 1.7%, and interassay variability was 20.6 $\pm$ 5.5%. DX600 was subsequently used in the assays for patient samples.

Patient sample collection. This study received institutional review board approval. Plasma samples (n = 228) were obtained from any subject suspected of having clinical HF and had a measured B-type natriuretic peptide (BNP) (ADVIA assay, Siemens Corporation, New York, New York) at the Cleveland Clinic between March and August 2006. We excluded patients who had congenital heart diseases, with acute coronary syndromes, or who were in the post-operative setting. Manual chart review was then performed by a physician blinded from the analysis to adjudicate both the presence of HF and its severity. Clinical, echocardiographic (within 100 days of sample isolation, including left ventricular ejection fraction [LVEF] assessment using biplane Simpson's rule), and biochemical characteristics and demographics were determined before database closure and blinding and before sample analysis.

Statistical analysis. Univariate analyses were conducted with the Student t test or chi-square test between groups and 1-way analysis of variance among sACE2 interquartile range for the various clinical parameters. Logistic regression analysis was performed to assess for confounding variables. Stepwise analysis was also performed in each of the models used (probability to enter or leave = 0.10), which yielded results similar to those presented. Missing data were left unassigned during the analysis (did not exceed n = 8 for any variable). All statistical analysis was performed with JMP 6.0.2 (SAS Institute, Cary, North Carolina). A p value <0.05 was considered significant.

Results

sACE2 activity measurement. The specific ACE2 inhibitor DX600 (8) inhibited recombinant ACE2 activity in a

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Abbreviations and Acronyms

ACE2 = angiotensin-converting enzyme 2

Ang = angiotensin

BNP = B-type natriuretic peptide

HF = heart failure

NYHA = New York Heart Association

RAAS = renin-angiotensin-aldosterone system

sACE2 = soluble angiotensin-converting enzyme 2

Table 1	Patient Demographics		

	Suspected HF	
Variables	**HF Present by Adjudication (n = 159)**	**No HF by Adjudication (n = 69)**
Mean age ± SD (yrs)	63.1 ± 14	59.1 ± 13
Men (%)	68	62
Mean LVEF ± SD (%)	34 ± 17*	57 ± 7
Mean BNP ± SD (pg/ml)	403 ± 552*	75 ± 80
Comorbid conditions		
Hypertension (%)	67%†	35%
Diabetes (%)	34%†	5%
Atrial fibrillation (%)	35%	64%†
Coronary artery disease (%)	54%†	19%
Creatinine ± SD (mg/dl)	1.3 ± 0.95*	1.02 ± 0.88
Medication use (%)		
ACE inhibitor	60%†	28%
Beta-blocker	72%†	50%
ARB	21%	13%
Loop diuretic	75%†	17%
Aldosterone antagonist	36%†	7%
Digoxin	38%†	12%

*$p < 0.001$, Student t test. †$p < 0.001$, chi-square test.

ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker; BNP = B-type natriuretic peptide; HF = heart failure; LVEF = left ventricular ejection fraction.

dose-dependent fashion (Fig. 1A). Both DX600 (1 μmol/l) and a polyclonal anti-ACE2 antibody (8 μg/ml) inhibited sACE2 activity in human plasma samples, demonstrating the observed increase in fluorescence was attributable specifically to sACE2 activity (Fig. 1B). Soluble angiotensin-converting enzyme 2 activity was inhibited by DX600 in commercially available plasma, indicating the ubiquitous basal expression of sACE2 (13.7 ± 3.6 ng/ml).

Study population and sACE2 activity. In the study cohort, 70% had a clinical diagnosis of HF (cases), and 30% showed no biochemical or clinical evidence of HF (controls) (Table 1). Plasma sACE2 activity was stratified by quartiles. Increasing sACE2 activity was strongly correlated with a diagnosis of HF, irrespective of etiology, and was not associated with any other disease state (Table 2). The odds ratio of predicting HF with sACE2 values above the fourth quartile was 4.8 (2.0 to 11.9, p = 0.0002, Fisher exact test). Soluble ACE2 activity also was associated with a worsening LVEF, increasing BNP levels, and use of loop diuretics (Table 2). The association between sACE2 activity and aldosterone antagonists was more clearly demonstrated by the use of a direct comparison (sACE2 ng/ml, 95% confidence interval [absence vs. presence of aldosterone antagonists]: 29.6 [26.2 to 32.9] vs. 39.3 [34.0 to 44.7], p = 0.0025, analysis of variance). In logistic regression analysis, sACE2 activity was independently associated with the presence of HF (Table 3).

Determinants of sACE2 activity. Greater sACE2 activity was associated with worsening New York Heart Association (NYHA) functional class (Fig. 2). Using logistic regression analysis, we found sACE2 activity to be independently associated with increasing NYHA functional class (p = 0.023) and log-transformed BNP (p = 0.003) and was inversely associated with LVEF (p = 0.023). Aldosterone antagonists were the only class of medication found to independently associate with sACE2 plasma activity (chi-square = 4.2, p = 0.040).

Table 2	Clinical Characteristics Based on Quartile Plasma sACE2 Activity			

Variable*	sACE2 Quartile 1	sACE2 Quartile 2 + 3	sACE2 Quartile 4	p Value†
sACE2 (ng/ml)	<16.6	16.6–41.1	>41.1	—
Age (yrs)	58.4 (54.8–62.1)	62.7 (60.2–65.3)	63.7 (60.0–67.3)	0.09
Gender (% male)	50.9	69.6	75.0	0.014
HF (%)	54.4	67.8	89.3	0.0002
Ischemic CM (%)	42.0	44.4	40.4	NS
LVEF (%)	48.9 (44.4–53.5)	41.1 (38.0–44.3)	32.0 (27.4–36.6)	<0.0001
BNP (pg/ml)	161 (42–280)	231 (147–315)	601 (480–721)	<0.0001
Creatinine (mg/dl)	1.07 (0.82–1.31)	1.23 (1.06–1.40)	1.46 (1.21–1.71)	0.08
Comorbid conditions (%)				
Coronary artery disease	26.4	36.5	51.2	0.14
Hypertension	54.4	53.0	69.6	0.10
Diabetes	22.8	25.2	26.7	0.88
Atrial fibrillation	40.4	40.0	55.4	0.14
Medications (%)				
Aldosterone antagonist	21.1	25.2	39.3	0.07
Loop diuretic	43.9	52.2	82.1	<0.0001
Beta-blocker	63.2	66.1	64.2	0.92
ACE inhibitor	49.1	50.4	51.8	0.96
ARB	15.8	20.1	17.9	0.78

*Continuous variables expressed as the mean (95% confidence interval). †Analysis of variance across sACE2 quartiles, chi-square test for nominal variables.

CM = cardiomyopathy; sACE = soluble angiotensin-converting enzyme; other abbreviations as in Table 1.

Discussion

Understanding the relationship between ACE2, cardiac cell growth, fibrosis, and remodeling may help us better understand the protective RAAS pathways involved in HF. Despite many elegant animal studies, there is a relative paucity of knowledge about whether ACE2 physiology is relevant in humans. Herein, we present 3 important observations in human HF: 1) sACE2 activity can be readily measured in heterogeneous patient populations; 2) sACE2 activity is elevated in human HF; and 3) sACE2 activity correlates independently with worsening disease severity as defined by LVEF and NYHA functional class.

One of the most intriguing questions our study raises is what role ACE2 plays in HF progression. Similar to its counterpart ACE, ACE2 is an integral membrane protein with a signal peptide and an N-terminal transmembrane domain. The presence of circulating ACE2 in plasma indicates either the cause or effect of an adaptive or maladaptive physiologic process operative in human HF. Unlike ACE, it is known that cleavage of membrane ACE2 into the soluble form is in part dependent on the tumor necrosis factor-α convertase ADAM17, a protease up-regulated in HF (9,10). Up-regulation of proinflammatory cytokines and activation of proteases during HF has long been implicated as a pathologic mechanism of cardiac remodeling (9,11). Therefore, cleavage of membrane ACE2 may be due to the up-regulation of a pathological protease, resulting in a relative decrease in local membrane ACE2 levels and cardiac dysfunction. Alternatively, cleavage and release of membrane ACE2 as a soluble form may offer a compensatory mechanism in HF. Angiotensin 1-7 has a very short half-life (<9 s), and release of sACE2 from the vascular endothelium may serve to alter systemic Ang(1-7)
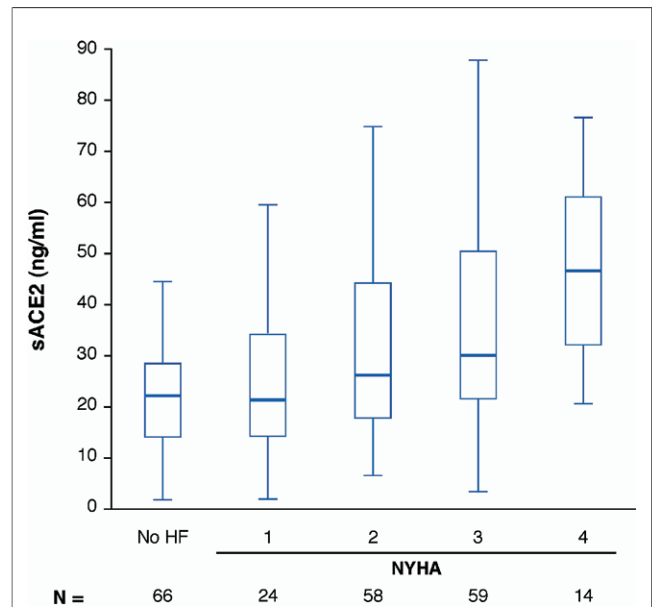

Figure 2   **sACE2 Activity Was Associated With Worsening NYHA Functional Class**

Quartile plots are shown. One-way analysis of variance, p = 0.0067. Number of patients (n) is given for each group. HF = heart failure; NYHA = New York Heart Association; sACE2 = soluble angiotensin-converting enzyme 2 activity.

concentrations and the relative peripheral balance of ACE2/ACE. By demonstrating the relationship between greater levels of sACE2 activity and HF, these data give us insights into the systemic activation of an endogenous counter-regulatory pathway beyond its membrane-bound confinements.

ACE2 expression can be modulated through a variety of mechanisms. Aldosterone decreases ACE2 transcription through a nicotinamide adenine dinucleotide phosphate oxidase-mediated pathway (12). It is therefore interesting to observe the direct correlation between the use of aldosterone receptor antagonists and greater sACE2 activity, which is consistent with the observation of increased ACE2 production from patients treated with spironolactone, indicating a potential mechanism of their beneficial effects (12). Stimulation through the AT-1 receptor is also known to suppress ACE2 expression (13). However, unlike animal studies, patients in our study were exposed to long-term inhibition of the RAAS, which might account for the lack of association between plasma sACE2 and the use of either ACE inhibitors or Ang receptor blockers.

Association between plasma sACE2 activity and plasma BNP provides an objective validation of the association between the systemic release of ACE2 and HF severity. As the detection of sACE2 activity likely signifies the activation of counter-regulatory mechanisms, the ultimate clinical utility of measuring sACE2 activity may depend on our ability to possibly target activation of the ACE2–Ang(1-7)–Mas pathway rather than to improve the clinical diagnosis of HF in the acute setting, the latter task in which plasma

Table 3	**Univariate and Multivariate HF Prediction Model Based on Plasma sACE2 Activity**	
Variable	**OR (95% CI)**	**p Value**
sACE2 >41.1 (ng/ml)	4.8 (2.0–11.9)	<0.0001
Adjusted for age (yrs)	4.7 (2.0–12.8)	0.0001
Adjusted for gender (% male)	4.7 (2.1–12.9)	<0.0001
Adjusted for LVEF (%)	4.9 (1.6–18.0)	0.009
Adjusted for BNP >100 (pg/ml)	3.1 (1.3–8.7)	0.03
Adjusted for creatinine (mg/dl)	4.2 (1.8–11.5)	0.002
Adjusted for coronary artery disease	4.6 (1.9–12.8)	0.001
Adjusted for hypertension	4.3 (1.8–11.8)	0.002
Adjusted for atrial fibrillation	6.9 (2.8–19.4)	<0.0001
Adjusted for aldosterone antagonists	4.3 (1.8–11.9)	0.002
Multivariable model: limited*		
sACE2 >41.1 (ng/ml)	4.2 (1.3–15.7)	0.03
Multivariable model: full†		
sACE2 >41.1 (ng/ml)	6.4 (1.5–32.2)	0.02

*Parameters included in the limited clinical model include age, gender, BNP >100 ng/ml, LVEF, and creatinine. †Parameters included in the full model include those in the limited model in addition to the use of loop diuretics, aldosterone antagonists, ACE inhibitors and ARBs, and the presence of atrial fibrillation, diabetes mellitus, hypertension, and coronary artery disease.

CI = confidence interval; OR = odds ratio; sACE = soluble angiotensin-converting enzyme; other abbreviations as in Table 1.

BNP testing has been well validated to perform. Our data support further investigations to better understand the clinical utility and significance of targeting ACE2 pathways for therapeutic interventions.

Conclusions

Using a specific and sensitive assay, soluble ACE2 activity can be detected in human plasma. Increased soluble ACE2 activity is associated with more advanced HF.

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Key Words: heart failure ■ ACE2 ■ hypertrophy ■ soluble.