Brain-Selective Overexpression of Angiotensin-Converting Enzyme 2 Attenuates Sympathetic Nerve Activity and Enhances Baroreflex Function in Chronic Heart Failure

Liang Xiao, Lie Gao, Eric Lazartigues, Irving H. Zucker

Abstract—Angiotensin-converting enzyme 2 (ACE2) has been suggested to be involved in the central regulation of autonomic function. During chronic heart failure (CHF), elevated central angiotensin II signaling contributes to the sustained increase of sympathetic outflow. This is accompanied by a downregulation of ACE2 in the brain. We hypothesized that central overexpression of ACE2 decreases sympathetic outflow and enhances baroreflex function in CHF. Transgenic mice overexpressing human ACE2 selectively in the brain (SYN-hACE2 [SA]) and wild-type littermates (WT) were used. CHF was induced by permanent coronary artery ligation. Four weeks after coronary artery ligation, both WT and SA mice exhibited a significant decrease in left ventricular ejection fraction (<40%). A slight decrease in mean arterial pressure was found only in SA mice. Compared with WT mice with CHF, brain-selective ACE2 overexpression attenuated left ventricular end-diastolic pressure; decreased urinary norepinephrine excretion; baseline renal sympathetic nerve activity (WT CHF: 71.6±7.6% max versus SA CHF: 49.3±6.1% max); and enhanced baroreflex sensitivity (maximum slope: WT sham: 1.61±0.16%/mm Hg versus SA CHF: 1.51±0.17%/mm Hg). Chronic subcutaneous blockade of mas receptor increased renal sympathetic nerve activity in SA mice with CHF (A779: 67.3±5.8% versus vehicle: 46.4±3.6% of max). An upregulation in angiotensin II type 1 receptor expression was detected in medullary nuclei in WT CHF mice, which was significantly attenuated in SA mice with CHF. These data suggest that central ACE2 overexpression exerts a potential protective effect in CHF through attenuating sympathetic outflow. The mechanism for this effect involves angiotensin (1-7) mas signaling, as well as a decrease in angiotensin II type 1 receptor signaling in the medulla. (Hypertension. 2011;58:1057-1065.) ● Online Data Supplement

Key Words: heart failure • angiotensin-converting enzyme 2 • angiotensin II • angiotensin I (1-7) • autonomic function • baroreflex

Hyperactivity of the sympathetic nervous system is a characteristic of the chronic heart failure (CHF) state. Although sympatho-excitation initially helps maintain cardiac output and arterial pressure, clinical and experimental evidence suggests that sympathetic activation becomes an important component that contributes to the deterioration in cardiac function in the long term.1–3 Angiotensin (Ang) II has a significant influence on cardiovascular function and sympathetic nerve activity via activation of central Ang II type 1 receptors (AT1Rs) in presynaptic nuclei, such as the rostral ventrolateral medulla (RVLM),4–6 the paraventricular nuclei,7,8 and the subfornical organ.9 A newly identified component of the renin-Ang system, Ang-converting enzyme 2 (ACE2), is a homolog of ACE, which cleaves the phenylalanine residue from the carboxy-terminus of Ang II and forms Ang (1-7).10 With weaker affinities to the AT1R and Ang II type 2 receptor (AT2R),11 Ang(1-7) preferentially binds to and stimulates an oncoreceptor called “mas.”12 Ang (1-7) has been shown to possess vasodilator activity in coronary vessels and exhibits opposing effects to stimulation by Ang II.13 Ang (1-7) negatively modulates Ang II/AT1R-activated c-Src and its downstream targets extracellular signal-regulated kinases 1/2 and NAD(P)H oxidase in endothelial cells.14 Mas receptor-knockout mice exhibit increased arterial pressure and reduced NO bioavailability.15 ACE2, Ang-(1-7), and the mas receptor are all found in the brain; however, their functions in the central nervous system are still controversial and not completely clear. Potts et al17 showed that, in anesthetized rabbits, Ang(1-7) possessed a sympatho-excitatory action when given into the RVLM, although at much higher doses than that of Ang II. Similar results were obtained by Fontes et al18 and by Silva et al19 in the rat. In a more recent study, Silva et al20 injected the mas receptor antagonist A-779 in the paraventricular nuclei of anesthetized rats and observed a decrease in renal sympathetic nerve activity (RSNA), suggesting that Ang-(1-7) is...
sympatho-excitatory, although transiently compared with Ang II. In contrast, studies by Ferrario et al suggest a sympatho-inhibitory effect of Ang-(1-7). Gironacci et al showed that Ang-(1-7) decreased norepinephrine (NE) release from the hypothalamus of spontaneous hypertensive rats. Yamazato et al reported a 40% decrease in ACE2 in the RVLM of spontaneous hypertensive rats compared with Wistar-Kyoto control rats. These investigators bilaterally overexpressed ACE2 in the RVLM, which resulted in a decrease in blood pressure (BP) in spontaneous hypertensive rats. Recently, Feng et al showed that ACE2 overexpression in a subfornical organ decreased AT1R expression and the pressor response to central Ang II in mice. In addition, this group also showed that brain-specific ACE2 overexpression attenuates neurogenic hypertension after subcutaneous Ang II infusion.

Recent data from our laboratory indicate that, in the CHF state, central expressions of ACE and ACE2 are reciprocally related. To assess the role of central ACE2 in the regulation of BP and sympathetic function in CHF, we hypothesized that overexpression of ACE2 in the brain decreases sympathetic outflow and improves baroreflex sensitivity in mice with coronary artery ligation-induced CHF.

Materials and Methods
An expanded Materials and Methods section is available in the online Data Supplement (please see http://hyper.ahajournals.org).

Transgenic Mice and Animal Husbandry
Experiments were performed on 3-month–old male SYN-hACE2 (SA) transgenic mice and wild-type nontransgenic (WT) littermates. SA transgenic mice were generated as described previously. All of the mice were housed in standard polypropylene cages and placed in a temperature and humidity-controlled facility. Mice were maintained on a 12:12-hour light-dark cycle (6:00 AM to 6:00 PM lights on) and fed with standard mouse chow (Harlan Laboratories, Indianapolis, IN). Water was available ad libitum. All of the experiments were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and conformed with the National Institutes of Health Guide for Laboratory Animal Use.

CHF Model
CHF was generated by permanent coronary artery ligation (CAL) with a method modified from that described previously. Four weeks after the CAL, echocardiography was carried out using a Visual Sonics VEVO 770 40 MHz echocardiographic system. Mice were anesthetized under light isoflurane anesthesia on a heated table with core temperature constantly monitored. The cardiac ejection fraction, fractional shortening, and cardiac chamber dimensions and volumes were measured and calculated. Mice with an ejection fraction <45% were considered to be in CHF.

Arterial Pressure and Heart Rate Recording in the Conscious State
After the echocardiography was performed, a radiotelemetry unit (PA-C10, Data Science International, St Paul, MN) was implanted. After 1 week of recovery, the telemeters were activated and arterial pressure was recorded 10 minutes every hour for 3 days at a sampling rate of 1 kHz.

Spontaneous Baroreflex Sensitivity
Spontaneous baroreflex sensitivity (SBRS) was calculated using the sequence method, as described previously. In brief, with hemodynamic tracings loaded into the HemoLab Analyzer program, an R value of 0.8 was used for inclusion, and a delay of 0 or 3 beats between the arterial pressure values and the pulse interval values was selected as described in Laude et al.

Urinary NE Enzyme-Linked Immunoassay
A subgroup of animals was housed individually in metabolic cages (Harvard Apparatus, Holliston, MA) for 8 days. Daily food and water intake were measured. Urine volume and fecal weight were also measured daily. Urinary NE concentration was measured with a commercially available enzyme-linked immunoassay kit (Rocky Mountains Diagnostics, Colorado Springs, CO). Total NE excretion for each day was calculated from NE concentration multiplied by daily urine volume. Animals were acclimated to the metabolic cage for 5 days. The average values from each animal over days 6 to 8 were used for statistical comparison.

Implantation of Osmotic Minipump for Drug Infusion
In some of the mice, a 7-day ALZET osmotic minipump (model 2001; Durect Corporation, Cupertino, CA) was implanted subcutaneously in the dorsal neck region for systemic infusion of saline or the mas receptor antagonist, A-779, at a rate of 200 ng·kg⁻¹·min⁻¹. After 7 days of infusion, the animals were used for RSNA recording.

RSNA Recording
After the echocardiography was performed, acute RSNA recordings were performed in a subset of animals. The baroreflex function was analyzed by logistic regression over the entire pressure range after IV injection of phenylephrine. The values of BP, heart rate (HR), and RSNA were acquired every 2 seconds from the threshold to the saturation points. As described previously, the mean values for each curve parameter were used to derive composite curves for each experimental group.

Western Blotting Analysis
After euthanasia, brains were quickly removed from the animals and immediately frozen on dry ice. Tissue samples from cortex, nucleus tractus solitarii (NTS), and RVLM were punched in a cryostat according to coordinates taken from a mouse atlas. Tissue lysates were processed against AT1R, AT2R, mas, ACE, and ACE2 antibodies. Bands were visualized using an enhanced chemiluminescence system, quantified with ImageJ software, and normalized with GAPDH.

Statistical Analyses
All of the data are expressed as mean±SE. Student t test was used when comparing 2 groups. A 2-way ANOVA with the Bonferroni post hoc test was used for analyzing the differences between multiple groups. Prism 5 (GraphPad Software, Inc, San Diego, CA) software was used for statistical analysis. A P value of <0.05 was taken as indicative of statistical significance.

Results

CHF in Mice
Four weeks after CAL surgery, both SA mice and WT control mice exhibited signs of impaired cardiac function, as measured by echocardiography. As shown in the Table, ejection fraction and fractional shortening were significantly lower in CHF animals than in sham animals. However, no significant differences were found between SA and WT mice. Left ventricular end diastolic pressure was markedly increased in WT CHF animals compared with their sham counterparts, whereas SA mice also exhibited a significant increase in left ventricular end diastolic pressure during CHF that was significantly less than in WT animals. Declines in ±dp/dt max, an indicator of myocardial contractility and relaxation,
were observed in both SA and WT mice to a similar extent during CHF.

Central Overexpression of ACE2 Decreases BP in Mice With CHF

BP and HR in the conscious state were monitored with radiotelemetry 1 week after catheter and transmitter implantation (5 weeks after CAL). As shown in Figure 1, the baseline values of mean arterial pressure in 24 hours in SA CHF mice were significantly lower than in SA sham mice (90.8 ± 4.4 mm Hg, n = 7, versus 109.4 ± 3.7 mm Hg, n = 8; P < 0.05). However, there was no statistical significance in mean arterial pressure between WT CHF mice (100.2 ± 8.2 mm Hg; n = 7) and WT sham animals (105.3 ± 3.6 mm Hg; n = 9). There was no significant difference in HR between groups.

Central Overexpression of ACE2 Decreases NE Excretion in CHF

Given the evidence that central ACE2 overexpression decreased left ventricular end diastolic pressure and BP in mice with CHF, we examined the role of central ACE2 overexpression on sympathetic function. We hypothesized that central ACE2 overexpression decreases peripheral resistance through attenuation of sympathetic nerve activity. As an indirect index of sympathetic tone in conscious mice, urinary NE excretion was measured 4 weeks after CAL and after 5 days of acclimatization to the metabolic cage. Similar to previous studies,34,36 urinary NE excretion was significantly elevated after CAL (623 ± 72 ng/d, n = 7, versus WT sham 377 ± 57 ng/d, n = 10; P < 0.05). However, SA mice did not show a significant increase in NE after CHF (SA CHF 378 ± 39 ng/d, n = 6, versus SA sham 286 ± 30 ng/d, n = 8; P < 0.05; Figure 2).

Table. Characteristic Cardiac Findings in SYN-hACE2 Mice With CHF

<table>
<thead>
<tr>
<th>Cardiac Function</th>
<th>Wild-Type Sham</th>
<th>Wild-Type CHF</th>
<th>SYN-hACE2 Sham</th>
<th>SYN-hACE2 CHF</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF, %</td>
<td>60.6 ± 1.4</td>
<td>37.0 ± 2.4*</td>
<td>55.5 ± 2.6</td>
<td>37.0 ± 2.3*</td>
</tr>
<tr>
<td>FS, %</td>
<td>32.1 ± 1.0</td>
<td>17.8 ± 1.2*</td>
<td>28.6 ± 1.6</td>
<td>20.1 ± 2.3*</td>
</tr>
<tr>
<td>SV, μL</td>
<td>43.1 ± 2.9</td>
<td>39.7 ± 8.9</td>
<td>43.7 ± 7.4</td>
<td>37.3 ± 3.6</td>
</tr>
<tr>
<td>CO, mL/min</td>
<td>19.3 ± 1.3</td>
<td>16.3 ± 4.0</td>
<td>19.2 ± 3.1</td>
<td>15.6 ± 1.8</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>3.7 ± 0.9</td>
<td>15.3 ± 2.7*</td>
<td>−0.4 ± 0.5</td>
<td>7.4 ± 2.2†</td>
</tr>
<tr>
<td>+ dp/dt max, mm Hg/s</td>
<td>7027 ± 705</td>
<td>3193 ± 665*</td>
<td>7539 ± 1713</td>
<td>3513 ± 293*</td>
</tr>
<tr>
<td>− dp/dt max, mm Hg/s</td>
<td>−6141 ± 1024</td>
<td>−2419 ± 475*</td>
<td>−7826 ± 1949</td>
<td>−3331 ± 256*</td>
</tr>
</tbody>
</table>

Echocardiographic results and hemodynamic parameters: EF indicates ejection fraction; FS, fractional shortening; SV, stroke volume; CO, cardiac output; LVEDP, left ventricle end-diastolic pressure. CHF indicates chronic heart failure. Each value represents mean ± SE (n = 7 to 12).

*P < 0.05 vs sham operation.
†P < 0.05 vs wild-type CHF animal.

Figure 1. Twenty-four–hour mean arterial pressure (MAP) tracing (A), baseline MAP (B), and heart rate (C) from wild-type (WT) and SYN-hACE2 (SA) mice with either sham surgery or chronic heart failure (CHF) in conscious state. *P < 0.05 vs the corresponding group of sham animals, n = 7 to 9 per group.
Central Overexpression of ACE2 Decreases RSNA in CHF

To examine the effects of central ACE2 overexpression on sympathetic outflow in mice with CHF, we recorded RSNA in anesthetized mice. Figure 3A shows original recordings from a representative mouse in each group. Although these tracings are from different animals studied under similar conditions, spike frequency is clearly increased in the WT CHF mouse compared with the WT sham mouse. The SA CHF mouse exhibited less activity than the WT CHF mouse. As shown in Figure 3B, similar baseline RSNA values were observed in WT sham and SA sham animals. RSNA in WT CHF mice were significantly increased compared with WT sham controls (WT CHF: 71.6 ± 7.6% versus WT sham: 36.1 ± 4.3% of max; P < 0.05). In SA animals, although there was also a significant increase in RSNA in CHF mice compared with sham animals (SA CHF: 49.3 ± 6.1% versus SA sham: 32.0 ± 4.9% of max; P < 0.05), the value was significantly lower than in WT CHF mice. To address the roles of Ang (1-7) and the mas receptor in SA mice, the mas receptor antagonist A779 was used. As shown in Figure 3C, compared with vehicle infusion, A779 significantly increased RSNA in SA mice with CHF (A779: 67.3 ± 5.8% versus vehicle: 46.4 ± 3.6% of max; P < 0.05).

Central Overexpression of ACE2 Improves Baroreflex Function in CHF

To study the role of ACE2 on BP and sympathetic nerve activity regulation in mice with CHF, baroreflex sensitivity was measured after IV injection of phenylephrine in anesthetized mice. In addition, the SBRS was evaluated using the sequence method. As shown in Figure 4A, after bolus injections of phenylephrine, arterial BP in each group of mice was increased to approximately the same extent (mean arterial pressure: ~140 mm Hg at peak). HR and RSNA were decreased in both WT and SA sham animals. Increasing BP resulted in a profound bradycardia and sympathoinhibition in WT sham and SA sham mice. On the other hand, WT CHF mice responded with very little change in HR or RSNA. SA CHF mice responded to the hypertensive stimulus with a larger bradycardia and sympathoinhibition compared with their WT counterparts. In CHF animals, the gains for both HR and RSNA were decreased compared with sham animals (HR gain max: WT CHF: 3.12 ± 0.72 versus WT sham 5.61 ± 0.24 bpm/mm Hg; SA CHF: 4.96 ± 0.56 versus SA sham 7.00 ± 0.51 bpm/mm Hg; P < 0.05; RSNA gain max: WT CHF: 0.73 ± 0.16 versus WT sham: 1.61 ± 0.16; SA CHF: 1.51 ± 0.17 versus SA sham: 1.96 ± 0.05; n = 4–5 per group; P < 0.05); however, the HR and RSNA gains of SA CHF mice were significantly higher than the gains of WT CHF mice (P < 0.05; Figures 4B, 4C, and S1, available in the online Data Supplement). Similarly data from SBRS analysis were collected from mice in the conscious state. As shown in Figure 5, there was no significant difference between WT sham (up-sequence: 2.01 ± 0.29 ms/mm Hg; down-sequence: 2.07 ± 0.34 ms/mm Hg; n = 8) and SA sham (up-sequence: 3.10 ± 0.37 ms/mm Hg; down-sequence: 2.05 ± 0.36 ms/mm Hg; n = 8) animals. SBRS was significantly decreased in WT CHF mice (up-sequence: 0.85 ± 0.10 ms/mm Hg; down-sequence: 0.84 ± 0.11 ms/mm Hg; n = 5, versus WT sham; P < 0.05). On the other hand, in SA CHF mice, SBRS shows a slight reduction 4 weeks after CAL (up-sequence: 2.36 ± 0.14 ms/mm Hg; down-sequence: 1.50 ± 0.15 ms/mm Hg; n = 7), which is significantly enhanced compared with WT CHF animals.

Central ACE2 Overexpression Normalizes Ang Receptor Expression in Brain Stem in CHF

Because both AT1R and AT2R have been implicated in the modulation of sympathetic activity and baroreflex function,37,38 we evaluated the expression of Ang receptors in SA mice with CHF (Figure 6). Samples were punched from NTS and RVLM. Western blot data revealed that the effect of CHF on AT1R expression in both NTS and RVLM depends on the mouse genotype. There was a statistically significant interaction between cardiac function and genotype. The post hoc analysis suggests that AT1R expression was upregulated in WT CHF mice in both NTS (WT CHF 0.16 ± 0.02 versus WT sham 0.051 ± 0.02; P < 0.05; Figure 6A and 6B) and RVLM (WT CHF 0.10 ± 0.01 versus WT sham 0.07 ± 0.08; P < 0.05; Figure 6D and 6E) in WT CHF mice, which is similar to our previous findings.37,38 This upregulation was attenuated in SA CHF mice in RVLM (SA CHF 0.06 ± 0.01 versus WT CHF; P < 0.05) and NTS (SA CHF 0.07 ± 0.02 versus WT CHF; P < 0.05). Although AT1R expression appears to be elevated in sham SA mice compared with WT, this was not significant. AT1R expression in NTS was decreased in WT CHF mice (WT CHF 0.20 ± 0.02 versus WT sham 0.35 ± 0.03; P < 0.05; Figure 6A and 6C). This decrease was not found in SA animals (SA CHF: 0.29 ± 0.03 versus SA sham: 0.37 ± 0.03; P = 0.40). Although no significant change was seen for AT2R expression in the RVLM between sham and CHF groups, AT2R expression was higher in SA animals compared with WT counterparts (SA: 0.56 ± 0.03 versus WT: 0.44 ± 0.03; P < 0.05).

We also investigated the expression of other renin-Ang system components. Consistent with our previous report,26 ACE was upregulated in both NTS and RVLM in WT mice during CHF (NTS: WT CHF 0.63 ± 0.05 versus WT sham 0.35 ± 0.06, P < 0.01, Figure S2A and S2B, n = 8 or 9 each; RVLM: WT CHF 0.13 ± 0.01 versus WT sham 0.09 ± 0.01, P < 0.05, Figures S2B and S3A, n = 8 each). A statistically significant interaction was found between cardiac function and genotype (P < 0.05); this upregulation was abolished in
SA animals (NTS: SA CHF 0.38±0.06 versus WT CHF, P<0.05; RVLM: SA CHF 0.08±0.01 versus WT CHF, P<0.05; Figures S2A, S2B, S3A, and S3B). As shown in Figures S2C, 2D, S3C, and 3D, transgenic human ACE2 expression was detected in both NTS and RVLM in SA animals at 120 kDa, and endogenous mouse ACE2 was detected in both WT and SA mice, at a lower molecular weight than the transgenic human ACE2. Comparing the expression of endogenous ACE2 in both NTS and RVLM, CHF animals had significantly less ACE2 expression than sham counterparts (NTS: CHF 0.12±0.01 versus sham 0.20±0.02, P<0.01, n=10 each; RVLM: CHF 0.046±0.004 versus sham 0.064±0.004, P<0.05, n=10 each, including 5 WT and 5 SA animals). The effect of different endogenous ACE2 levels on cardiac function did not depend on which genotype was present. As shown in Figure S2E, S2F, S3E, and S3F, a significant increase in mas receptor expression was detected between sham and CHF animals in both NTS and RVLM (NTS: CHF 0.69±0.06 versus sham 0.43±0.07, P<0.01, n=9 each; RVLM: CHF 0.27±0.03 versus sham 0.14±0.03, P<0.05, n=10 each, including 5 WT and 5 SA animals), and the differences do not depend on the mouse genotype.

**Discussion**

In the present study, we investigated the role of central ACE2 overexpression on sympathetic function during CHF in a mouse CAL infarction model. These data suggest that overexpression of ACE2 selectively in the brain lowered BP, decreased RSNA and NE excretion, and improved baroreflex...
function in animals with CHF. Furthermore, the increased expression of AT1R protein in the NTS and RVLM of WT mice with CHF was abrogated in CHF mice that overexpress ACE2.

An upregulation of AT1R expression and its signaling has been found to be directly associated with increased excitability of presympathetic neurons and attenuated inhibitory baroreflex signals, both of which contribute to the sustained hyperactivation of the sympathetic nervous system and accelerate the exacerbation of CHF.36,37 The effects of central ACE2 overexpression have been examined in animal models where the central renin-Ang system has been enhanced. For
instance, Yamazato et al\textsuperscript{23} reported a decrease in ACE2 expression in the spontaneous hypertensive rat. Our laboratory showed recently that ACE2 expression was reduced in the RVLM, the NTS, and in the paraventricular nuclei of rabbits with pacing-induced CHF\textsuperscript{26}. In hypertensive animal models, ACE2 overexpression in the subfornical organ and RVLM lowers BP and blunts the AT\textsubscript{1}R upregulation.\textsuperscript{23–25} CHF is a hyperangiotensinergic but normotensive or even hypotensive state. Similar to that observed in hypertensive models,\textsuperscript{25} brain-selective ACE2 overexpression also lowered BP in mice with CHF (Figure 1). The decrease in BP in SA CHF mice could be attributed either to a worsening of cardiac function or to a decrease in sympathetic nerve activity that is augmented in the CHF state. Because echocardiographic data showed no differences in cardiac output between groups of mice, we speculate that the decrease in BP was caused by a decrease in total peripheral resistance, which is strongly influenced by sympathetic outflow, especially in the CHF state.

Although previous studies with ACE2 overexpression implied attenuation in sympathetic outflow in hypertensive animals,\textsuperscript{23,25} there are few direct recordings of sympathetic nerve activity. In the present study we recorded RSNA from SA and WT mice with and without CHF. Central ACE2 overexpression reduced the augmented RSNA in mice with CHF by \begin{math} \approx 50\% \end{math}. NE excretion was increased in WT CHF mice but largely normalized in SA CHF mice. The daily total NE excretion was similar to that reported by Infanger et al\textsuperscript{36} and correlates with the differences seen in baseline RSNA recordings in the various groups.

This study was not designed to evaluate the precise cellular mechanism by which increased ACE2 evokes sympathetic outflow. Although ACE2 converts Ang II to Ang (1-7), it is not clear whether the effects of neuron-specific ACE2 overexpression are caused by an increase in the Ang (1-7)-mas receptor signaling or a decrease of Ang II-AT\textsubscript{1}R signaling. Systemic blockade of the mas receptor by chronic subcutaneous infusion of mas receptor antagonist A-779 significantly increased RSNA in SA mice with CHF, which suggests an inhibitory role of Ang (1-7)-mas receptor signaling in regulating sympathetic outflow. One could speculate...

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Angiotensin II type 1 and type 2 receptor expression in nucleus tractus solitarius (NTS; A through C) and rostral ventrolateral medulla (RVLM; D through F). *P<0.05 vs the corresponding group in sham mice; †P<0.05 vs the wild-type (WT)-chronic heart failure (CHF) group; n=5 to 7 in each group.}
\end{figure}
that tissue Ang (1-7) is increased in these mice and augments the levels of cellular and tissue NO through mas receptor stimulation. NO evokes sympatho-inhibition when administered into the RVLM, paraventricular nuclei, and NTS. Because A-779 was given by systemic infusion in our study, we cannot determine whether the effect of A-779 is a central effect and/or a peripheral effect. However, because ACE2 is only overexpressed in the central nervous system, an increase in Ang (1-7)-mas signaling in the brain is more likely to play a sympathetic-inhibitory role in SA mice during CHF. Our data suggest an upregulation of mas receptor protein in the NTS and RVLM in CHF. We speculate that the upregulation may be a compensatory response to the decrease in Ang (1-7) in CHF. On the other hand, the reduction in tissue Ang II may also contribute to the sympatho-inhibition, enhancement in baroreflex function, and reduction in AT1R expression. The fact that AT1R expression was reduced in the NTS also suggests that a reduction in Ang II signaling may contribute to the enhancement in baroreflex function. This may help explain why ACE2 overexpression does not completely restore autonomic dysfunction in mice with CHF.

Although the animal model used here evokes overexpression of the human ACE2 protein in neurons throughout the brain, more specific overexpression has been investigated using viral vectors targeted to autonomic areas of the brain. The present results are consistent with those reported by Feng et al., in which viral overexpression of ACE2 in the subfornical organ reduced responses to intracerebroventricular Ang II. Furthermore, these investigators clearly showed that transfection of neuro 2A cells with ACE2 adenovirus also caused a decrease in AT1R expression.

One question that arises in this work is why overexpression of central ACE2 does not improve cardiac function in CHF mice, because these mice should have lower Ang II signaling and lower central oxidative stress. Indeed, in a recent study by Lindley et al., it was shown that overexpression of CuZn SOD improved cardiac function in mice with myocardial infarctions. The answer to this is not clear. Both viral and transgenic overexpressions of proteins are fraught with issues related to the level of protein expression relative to physiological levels. The decrease in oxidative stress in this and another earlier study by Lindley et al. may be extremely large compared with any effect on oxidative stress in the present study. Furthermore, although we measured RSNA and excreted NE, we do not know the effect of this model on cardiac sympathetic nerve activity per se.

In summary, we have shown that, in mice with neuron-targeted ACE2 overexpression in the brain, establishment of CHF is associated with a reduction in sympathoexcitation and an enhancement in baroreflex function. Given that ACE is elevated in the brain of animals with CHF, these data strongly suggest that the balance between ACE and ACE2 and perhaps between Ang II and Ang (1-7) is a critical feature in establishing normal sympathetic outflow.

Perspectives

CHF is a syndrome that initiates a positive feedback cycle in which compensatory neurohumoral activation worsens the CHF state, which, in turn, further activates the various neurohumoral systems, thus continuing the vicious cycle that characterizes this disease. The central renin-Ang II system is pivotal in driving this maladaptation. Either inhibition of Ang II signaling or activation of the Ang (1-7) pathway should be beneficial in the CHF state by virtue of contributing to a decrease in sympathetic activation. The validity of this principle is pointed out by experiments described in the current study in which central overexpression of the enzyme that makes Ang (1-7) from Ang II evokes sympathoinhibition. This finding suggests that the ACE2-Ang (1-7)-mas receptor axis may provide novel therapeutic targets for interrupting the positive feedback in CHF.

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Disclosures

None.

References


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Brain-Selective Overexpression of ACE2 Attenuates Sympathetic Nerve Activity and Enhances Baroreflex Function in Chronic Heart Failure.

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Material and Methods

Generation of transgenic mice.

SYN-hACE2 transgenic mice were generated as described previously\(^1\). Briefly, a fusion transgene (SYN-hACE2) consisting of 4.4 kb of the rat synapsin promoter and a cDNA encoding the full open-reading frame of the human ACE2 gene (NCBI accession number: AF291820) was constructed.

Coronary artery ligation

Coronary artery ligation was performed with the method modified from described by Michael \textit{et al.}\(^2\). In brief, under 1.5~2\% isoflurane anesthesia, each mouse was artificially ventilated using a mechanical respirator (Mouse Ventilator MiniVent model 845, Hugo Sachs Elektronik; tidal volume: 150 μL; frequency: 200 breaths/min) throughout surgery. A left thoracotomy was performed through an incision on the fourth left intercostal space, and the heart exposed. In the CHF group, the left anterior descending branch of the coronary artery was permanently tied with a 7-0 suture between the pulmonary artery outflow tract and the left atrium as it exits the aorta. The sham animals underwent thoracotomy and manipulation of the heart, but the coronary artery was not ligated. After these procedures, the lungs were re-inflated and the thorax was closed. The trachea was extubated after the animals began to recover from anesthesia.

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After the echocardiography was performed, a radiotelemetry unit (PA-C10, Data Science International; St Paul, MN) was implanted with the catheter inserted into the aorta through left common carotid artery under 1.5~2\% isoflurane anesthesia and aseptic conditions\(^3\). HR was derived from the arterial pressure (AP) pulse using a PowerLab (model 8S; ADInstruments; Colorado Springs, CO) data acquisition system. After one week of recovery the telemeters were activated and AP was recorded 10 minutes every hour for 3 days at a sampling rate of 1 kHz.

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After the echocardiography was performed, acute RSNA recordings were performed in a subset of animals. In brief, under urethane (800 mg/kg IP) and \(α\)-chloralose (40 mg/kg IP) anesthesia the trachea was cannulated to facilitate mechanical respiration. The right carotid artery was dissected, and a transducer (Millar Instruments, Houston, TX, model: SPR-1000) was advanced to the ascending aorta for recording of AP. Heart rate was derived from the AP pulse using the cardiotachometer function in the PowerLab Chart system (ADInstruments). A femoral vein was cannulated with a microrenathane-25 catheter for the administration of phenylephrine (PE) or sodium nitroprusside (SNP). Body temperature was kept at 37°C with a heating pad.

A branch of the renal nerve was isolated retroperitoneally through a left flank incision. The renal sympathetic nerves were exposed and placed on a pair of recording platinum-iridium electrodes, and the nerve-electrode preparation was covered with a silicone gel. The renal sympathetic nerve activity (RSNA) was amplified and filtered (bandwidth: 30–3,000 Hz) using a Grass P55C preamplifier. The nerve signal was monitored on an oscilloscope. The signal from the oscilloscope was displayed on a computer, where it was rectified, integrated, sampled (1 kHz), and converted to a digital signal by the Powerlab data-acquisition system. After baseline recording of BP, HR and
RSNA, phenylephrine (PE) (0.2µg/µL in 20-40 µl) was given intravenously and the response was recorded for the analysis of baroreflex sensitivity. At the end of the experiment, the mouse was euthanized with an overdose of pentobarbital sodium (150 mg/kg). The maximum nerve activity (Max) occurred 1–2 min after the mouse was euthanized. Background noise levels for sympathetic nerve activity were recorded 15–20 min after the mouse was euthanized. Using the unit conversion of Powerlab Chart system, baseline nerve activity was taken as percent of Max after the noise level was subtracted.

The baroreflex function was analyzed by logistic regression over the entire pressure range after injection of PE iv. The values of BP, HR and RSNA were acquired every 2 seconds from the threshold to the saturation points. As previously described\textsuperscript{4,5}, a sigmoid logistic regression curve was fit to the data points using the following equation:

\[
\text{HR or RSNA} = A / \left[ 1 + \exp \left( B (\text{MAP} - C) \right) \right] + D,
\]

where A is the HR or RSNA range, B is the slope coefficient, MAP is the mean arterial pressure, C is the pressure at the midpoint of the range (BP50), and D is the minimum HR or RSNA. The peak slope (or maximum gain) was determined by taking the first derivative of the baroreflex curve and was calculated with the equation:

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\text{Gain max} = A(1) \times A(2) \times [1/4],
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**Western Blotting Analysis**

After euthanasia, brains were quickly removed from the animals, and immediately frozen on dry ice. Tissue from cortex, nucleus tractus solitarii (NTS) and RVLM were punched in a cryostat according to coordinates taken from a mouse atlas\textsuperscript{6}. Total protein was extracted with Radioimmunoprecipitation Assay Buffer, and equal amount of samples were loaded and separated by SDS-PAGE, and then electrophoretically transferred to PVDF membranes. The membranes were blocked in Tris-buffered saline solution containing 1% Tween 20 and 5% non-fat dried milk. Then the membranes were washed and incubated with primary antibodies. Primary antibodies were purchased from the indicated sources: rabbit polyclonal antibodies anti-ACE2 (sc-20998), anti-ACE (sc-20791), anti-AT\textsubscript{1}R (sc-1173), and anti-AT\textsubscript{2}R (sc-9040), mouse monoclonal antibody anti-GAPDH (sc-32233) from Santa Cruz Biotechnology, and rabbit polyclonal antibody anti-mas (AAR-013) from Alomone Labs. Membranes were thoroughly washed in Tris-buffered saline solution containing 1% Tween 20 and incubated with horseradish peroxidase–conjugated anti-rabbit secondary antibody (Thermo). Bands were visualized using an enhanced chemiluminescence system, and quantified with ImageJ software.

**References**


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Figure S1. Composite arterial baroreflex and gain curves of arterial baroreflex function for HR (A) and RSNA (B) in wild type sham, SYN-hACE2 sham, wild type CHF, and SYN-hACE2 CHF mice.
Supplemental Figure S2

A.  

Wild Type | SYN-hACE2
---|---
sham | sham | CHF | CHF

ACE

GAPDH

B.  

![Bar graph showing expression levels of ACE/GAPDH in Wild Type and SYN-hACE2 with sham and CHF conditions.]

C.  

Wild Type | SYN-hACE2
---|---
sham | sham | CHF | CHF

ACE2

GAPDH

C.  

![Graph showing expression levels of endogenous ACE2/GAPDH in Wild Type and SYN-hACE2 with sham and CHF conditions.]

D.  

![Graph showing expression levels of endogenous ACE2/GAPDH in Wild Type and SYN-hACE2 with sham and CHF conditions.]

120 kD transgenic human ACE2
90 kD endogenous mouse ACE2
Supplemental Figure S2 continue

E.

F.

**Figure S2.** ACE, ACE2 and mas receptor expression in nucleus tractus solitarius (NTS). *P<0.05 compared with the corresponding group in sham mice; #P<0.05 compared with the WT-CHF group; n= 8 or 9 for ACE, and n= 5 for ACE2, and n= 4 or 5 for mas in each group.
Supplemental Figure S3

A. 

B. 

C. 

D.
Supplemental Figure S3 continue

E.  

![Image of Western blot showing expression of Mas and GAPDH in different groups](image)

Wild Type sham  SYN-hACE2 sham  Wild Type CHF  SYN-hACE2 CHF

Mas  

GAPDH  

F.  

![Image of bar graph showing mas/GAPDH ratio](image)

**Figure S3.** ACE, ACE2 and mas receptor expression in nucleus rostral ventrolateral medulla (RVLM). *P<0.05 compared with the corresponding group in sham mice; #P<0.05 compared with the WT-CHF group; n= 8 for ACE, and n= 5 for ACE2, and n= 5 or 6 for mas in each group.
ONLINE SUPPLEMENT

Brain-Selective Overexpression of ACE2 Attenuates Sympathetic Nerve Activity and Enhances Baroreflex Function in Chronic Heart Failure.

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and
Department of Pharmacology & Experimental Therapeutics²
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Material and Methods

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**References**


Supplemental Figure S1

Figure S1. Composite arterial baroreflex and gain curves of arterial baroreflex function for HR (A) and RSNA (B) in wild type sham, SYN-hACE2 sham, wild type CHF, and SYN-hACE2 CHF mice.
Supplemental Figure S2

A. 

<table>
<thead>
<tr>
<th></th>
<th>Wild Type sham</th>
<th>SYN-hACE2 sham</th>
<th>Wild Type CHF</th>
<th>SYN-hACE2 CHF</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. 

![Graph showing ACE/GAPDH ratio for Wild Type and SYN-hACE2 groups.]

C. 

<table>
<thead>
<tr>
<th></th>
<th>Wild Type sham</th>
<th>SYN-hACE2 sham</th>
<th>Wild Type CHF</th>
<th>SYN-hACE2 CHF</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D. 

![Graph showing endogenous ACE2/GAPDH ratio for Wild Type and SYN-hACE2 groups.]

* P<0.05

120 kD transgenic human ACE2
90 kD endogenous mouse ACE2
**Figure S2.** ACE, ACE2 and mas receptor expression in nucleus tractus solitarius (NTS). *P*<0.05 compared with the corresponding group in sham mice; #P*<0.05 compared with the WT-CHF group; n= 8 or 9 for ACE, and n= 5 for ACE2, and n= 4 or 5 for mas in each group.
Supplemental Figure S3

A. Wild Type sham | SYN-hACE2 sham | Wild Type CHF | SYN-hACE2 CHF

ACE

GAPDH

195kD

B.

ACE/GAPDH

* 0.16

0.14

0.12

0.10

0.08

0.06

0.04

0.02

0.00

Wild Type SYN-hACE2

Sham CHF

C. Wild Type sham | SYN-hACE2 sham | Wild Type CHF | SYN-hACE2 CHF

ACE2

120 kD transgenic human ACE2

90 kD endogenous mouse ACE2

GAPDH

D.

endogenous ACE2/GAPDH

0.08

0.06

0.04

0.02

0.00

Wild Type SYN-hACE2

Sham CHF

P<0.05
Supplemental Figure S3 continue

E.

![Image of Western blot analysis showing expression of Mas and GAPDH in different groups.

F.

![Image of bar graph showing expression levels of mas/GAPDH in wild type and SYN-hACE2 groups under sham and CHF conditions.

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