Brain-Selective Overexpression of Human Angiotensin-Converting Enzyme Type 2 Attenuates Neurogenic Hypertension

Yumei Feng, Huijing Xia, Yanhui Cai, Carmen M. Halabi, Lenice K. Becker, Robson A.S. Santos, Robert C. Speth, Curt D. Sigmund, Eric Lazartigues

Rationale: Angiotensin converting enzyme type 2 (ACE2) is a new member of the brain renin-angiotensin system, that might be activated by an overactive renin-angiotensin system.

Objective: To clarify the role of central ACE2 using a new transgenic mouse model with human (h)ACE2 under the control of a synapsin promoter, allowing neuron-targeted expression in the central nervous system.

Methods and Results: Syn-hACE2 (SA) transgenic mice exhibit high hACE2 protein expression and activity throughout the brain. Baseline hemodynamic parameters (telemetry), autonomic function, and spontaneous baroreflex sensitivity (SBRS) were not significantly different between SA mice and nontransgenic littermates. Brain-targeted ACE2 overexpression attenuated the development of neurogenic hypertension (Ang II infusion: 600 ng/kg per minute for 14 days) and the associated reduction of both SBRS and parasympathetic tone. This prevention of hypertension by ACE2 overexpression was reversed by blockade of the Ang-(1-7) receptor (D-Ala7-Ang-(1-7); 600 ng/kg per minute). Brain angiotensin II type 2 (AT2)/AT1 and Mas/AT1 receptor ratios were significantly increased in SA mice. They remained higher following Ang II infusion but were dramatically reduced after Ang-(1-7) receptor blockade. ACE2 overexpression resulted in increased NOS and NO levels in the brain, and prevented the Ang II–mediated decrease in NOS expression in regions modulating blood pressure regulation.

Conclusions: ACE2 overexpression attenuates the development of neurogenic hypertension partially by preventing the decrease in both SBRS and parasympathetic tone. These protective effects might be mediated by enhanced NO release in the brain resulting from Mas and AT2 receptor upregulation. Taken together, our data highlight the compensatory role of central ACE2 and its potential benefits as a therapeutic target for neurogenic hypertension. (Circ Res. 2010;106:373-382.)

Key Words: carboxypeptidase ■ blood pressure ■ nitric oxide ■ baroreflex ■ autonomic function

The renin–angiotensin system (RAS) is well known for its physiological and pathophysiological roles in the regulation of blood pressure (BP) and cardiovascular function.1,2 A new component of the RAS, angiotensin-converting enzyme (ACE) type 2 has been identified, from human heart failure ventricle and lymphoma cDNA libraries (reviewed elsewhere3,4). Although the angiotensin-converting enzyme type 2 (ACE2) transcript was first described in heart, kidney and testis, additional studies reported ACE2 mRNA in rat medulla oblongata4 and ACE2 activity in mouse brain.5 Recently, we showed the presence of both ACE2 protein and mRNA widespread throughout the murine brain, in regions involved in the central regulation of cardiovascular function as well as noncardiovascular regions.6 ACE2 converts Ang II into the vasodilatory peptide Ang-(1-7) with an affinity 400-fold higher than for Ang I.7 In the central nervous system (CNS), Ang-(1-7) has been shown to enhance sensitivity of the bradycardic component of the cardiac baroreceptor reflex8 and to promote vasodilation in hypertensive animals.5,10 As a key enzyme in generating Ang-(1-7), ACE2 is thought to be a pivotal player in central BP regulation.5,5

Several evidences from various laboratories have shown the beneficial effects of peripheral ACE2 in the regulation of cardiovascular hypertrophy and BP control.10–12 In the CNS, using a lentivirus coding for ACE2, Yamazato et al previously showed that ACE2 overexpression in the rostral ventrolateral medulla, could reverse hypertension in spontaneously hypertensive rats (SHR).13 More recently, we reported that brain-targeted ACE2 overexpression in the subfornical organ (SFO) prevents the acute Ang II–mediated pressor and...
drinking responses. However, because of the short-term expression and the low efficiency of the virus vectors, these acute studies could not address the long-term effects of ACE2 expression. This is particularly important as previous studies using transgenic mice overexpressing ACE2 selectively in the heart, resulted in lethal alterations of cardiac rhythm. Accordingly, the role of ACE2 in the central regulation of BP and the effects of chronic ACE2 overexpression on the development of hypertension need further investigation.

To achieve this goal, we developed a new transgenic mouse model with neuron-targeted overexpression of human (h)ACE2 in the CNS. Our data suggest that chronic ACE2 overexpression in syn-hACE2 (SA) mice impairs the development of neurogenic hypertension partially by preventing the decreases in baroreflex sensitivity and parasympathetic tone. Importantly, these protective effects may involve AT2 and Mas receptors upregulation, thus leading to increased NO signaling pathways and NO levels in the cerebrospinal fluid.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Generation of Transgenic Mice**

The SA fusion transgene (Figure 1A) was constructed and microinjected into fertilized C57BL/6JxSJL/J (B6SJLF2) mouse embryos at the University of Iowa Transgenic Animal Facilities. All mice were fed standard mouse chow and water ad libitum. All procedures were approved by Institutional Animal Care and Use Committees at the University of Iowa and Louisiana State University Health Science Center.

**RNA Isolation and RT-PCR**

Total RNA was isolated from various tissues from both transgenic and nontransgenic (NT) mice. Specific primers for hACE2 and β-actin were designed using PrimerQuest Software (IDT, Coralville, Iowa).

**Western Blot**

Tissue lysates (10 µg) from brain cortex, hypothalamus and brainstem were collected separately and processed against hACE2 antibodies. Specific bands were detected by chemiluminescence and quantitated by laser densitometry.

**ACE2 Activity**

ACE2 activity was measured in tissue lysates from brain cortex, hypothalamus and brainstem. Data are presented as amounts of substrate FPSVI converted to product per minute and are normalized for total protein.

**Immunohistochemistry**

Brains sections were processed for hACE2, AT1 and AT2 receptors, Mas protooncogene, endothelial NO synthase (eNOS), Ser1177-phosphorylated eNOS, Thr495-phosphorylated eNOS, and neuronal NOS (nNOS) detection.

**NO Measurement**

Mice were anesthetized and NO measured using a Free Radical Analyzer. The NO probe was positioned into the mouse lateral ventricle and the current measured following stabilization.

**Ang II and Ang-(1-7) Levels**

Brain regions and blood samples were collected and processed for Ang II and Ang-(1-7) measurement by the Wake Forest University Hypertension Core Laboratory.

**Physiological Recordings**

Male SA and control littermates (n=12), 8 to 10 weeks old, were instrumented with radiotelemetry probes. Following recovery, base-line BP was recorded for 4 days. Mice were then infused subcutaneously for 14 days using osmotic minipumps (Alzet) containing one of the following: (1) saline; (2) Ang II (600 ng/kg per minute), a model for neurogenic hypertension; (3) d-Ala7-Ang-(1-7) (600 ng/kg per minute), an Ang-(1-7) antagonist; or (4) Ang II plus d-Ala7-Ang-(1-7). Water intake was recorded daily. Spontaneous baroreflex sensitivity (SBRS) and autonomic function were also assessed.

**Statistical Analysis**

Data are expressed as means±SEM. Data were analyzed by Student’s t test or 2-way ANOVA (Bonferroni post hoc tests to compare replicate means) when appropriate. Statistical comparisons were performed using Prism5 (GraphPad Software, San Diego, Calif). Differences were considered statistically significant at P<0.05.

**Results**

**Characterization of Transgenic Mice**

Using standard transgenic technology, we generated 11 SA founders expressing the hACE2 transgene (Figure 1A). All founders were successfully bred to establish transgenic lines. Out of these founders, line 10 exhibited the highest level of transgene expression, whereas others exhibited moderate or low levels of hACE2 mRNA in the brain (Figure 1B). Using RT-PCR, we detected high level expression of the hACE2 transgene in the brains from line 10, but not in other tissues, confirming the brain specificity of the synapsin promoter (Figure 1C). To determine whether SA transgene expression was also reflected at the protein level, Western blotting was performed on brains from SA (line 10) and NT mice using a specific hACE2 antibody (Figure 1D). Expression of hACE2
protein (120 kDa) was detected in different brain regions in SA (ie, cortex, hypothalamus, and brainstem) but not in NT mice. To verify whether transgenic expression of the hACE2 protein is functional, ACE2 activity was assessed in both genotypes. As shown in Figure 1E, SA have significantly higher (10 to 15-fold; \( P < 0.001 \)) brain ACE2 activity compared to NT mice harboring only the endogenous ACE2 gene.

Using immunohistochemistry, we examined the distribution of transgene expression in the brain of SA mice. Widespread hACE2 immunostaining was detected throughout the CNS in cardiovascular regions like the SFO, nucleus of tractus solitarius (NTS) and rostral ventrolateral medulla (RVLM) (Figure 1J through 1L), as well as in noncardiovascular regions (Online Figure I). Very low levels were detected in NT mice (Figure 1F through 1H), suggesting weak mouse ACE2 cross-reactivity for this hACE2 antibody. Higher magnification revealed neuron-targeted hACE2 immunostaining (Figure 1M), consistent with the synapsin promoter specificity. Sections incubated without primary antibody showed a complete lack of immunostaining from both transgenic and NT mice (data not shown) confirming the specificity of staining.

Finally, to address the consequences of ACE2 overexpression on Ang peptides, Ang II and Ang-(1-7) were measured in the cortex, hypothalamus, and brainstem of control and transgenic mice (Table 1). Ang II was significantly reduced in the brainstem of SA (7.0 ± 1.1 pg/mg protein, \( n=12; P<0.05 \)) compared to NT (10.1 ± 1.0 pg/mg protein, \( n=12 \)) mice. Moreover, the balance between Ang II and Ang-(1-7) peptides was significantly altered in both hypothalamus and brainstem of SA mice, in favor of Ang-(1-7). Plasma peptides were not significantly different (Table 1).

**ACE2 Overexpression Reverses the Development of Neurogenic Hypertension**

To determine the functional consequences of hACE2 expression in SA mice, we first assessed the integrity of the BP response following acute intracerebroventricular Ang II (200 ng/200 nL) administration in conscious freely moving mice. Expression of exogenous hACE2 blunted the Ang II–mediated pressor response in SA mice (3±2 mm Hg, \( n=5 \)) compared to their NT littermates (15±3 mm Hg, \( n=5; P<0.05 \)), consistent with our previous report showing that adenovirus-mediated ACE2 overexpression prevents the pressor response to acute intracerebroventricular Ang II.\(^5\) To examine whether this functional expression of hACE2 in the brain can prevent the development of hypertension, we chronically infused mice, via the osmotic minipump, with a
Table 1. Baseline Angiotensin Peptides Levels in the Brain and Plasma

<table>
<thead>
<tr>
<th></th>
<th>NT</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cortex (pg/mg protein)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ang-II</td>
<td>8.1±0.4</td>
<td>7.1±0.7</td>
</tr>
<tr>
<td>Ang-(1-7)</td>
<td>7.1±1.1</td>
<td>6.6±0.9</td>
</tr>
<tr>
<td>Ang-II/Ang-(1-7)</td>
<td>1.7±0.2</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>Ang-(1-7)/Ang-II</td>
<td>0.6±0.1</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td><strong>Hypothalamus (pg/mg protein)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ang-II</td>
<td>11.0±1.8</td>
<td>8.6±1.6</td>
</tr>
<tr>
<td>Ang-(1-7)</td>
<td>7.7±1.3</td>
<td>10.0±1.2</td>
</tr>
<tr>
<td>Ang-II/Ang-(1-7)</td>
<td>1.4±0.2</td>
<td>0.9±0.2*</td>
</tr>
<tr>
<td>Ang-(1-7)/Ang-II</td>
<td>0.7±0.1</td>
<td>1.2±0.1*</td>
</tr>
<tr>
<td><strong>Brainstem (pg/mg protein)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ang-II</td>
<td>10.1±1.0</td>
<td>7.0±1.1*</td>
</tr>
<tr>
<td>Ang-(1-7)</td>
<td>13.8±1.7</td>
<td>14.9±1.5</td>
</tr>
<tr>
<td>Ang-II/Ang-(1-7)</td>
<td>0.7±0.1</td>
<td>0.5±0.1*</td>
</tr>
<tr>
<td>Ang-(1-7)/Ang-II</td>
<td>1.4±0.2</td>
<td>2.1±0.2*</td>
</tr>
<tr>
<td><strong>Plasma (pg/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ang-II</td>
<td>195±55</td>
<td>154±56</td>
</tr>
<tr>
<td>Ang-(1-7)</td>
<td>264±51</td>
<td>191±45</td>
</tr>
<tr>
<td>Ang-II/Ang-(1-7)</td>
<td>0.7±0.2</td>
<td>0.8±0.3</td>
</tr>
<tr>
<td>Ang-(1-7)/Ang-II</td>
<td>1.4±0.3</td>
<td>1.2±0.3</td>
</tr>
</tbody>
</table>

Data represent the Ang II, Ang-(1-7) levels and their ratios. Values are expressed as means±SEM. *P<0.05 vs NT mice.

slow-pressor dose of Ang II (600 ng/kg per minute for 14 days) and monitored BP changes in conscious freely moving animals using telemetry. The 24-hour baseline hemodynamic parameters and activity data are summarized in Online Table I. BP was not significantly different between genotypes (SA: 101±2.0; NT: 97.2±2.2 mm Hg; *P>0.05), indicating that mice with brain-targeted ACE2 overexpression remain normotensive. Saline infusion did not alter BP level in any of the genotypes (Figure 2A). Chronic Ang II infusion significantly increased BP in NT mice, reaching a hypertensive level after 14 days (132±5 mm Hg, n=12) compared to saline-infused controls (95±1 mm Hg, n=6; *P<0.05) (Figure 2A). Interestingly, SA mice exhibited a biphasic pattern. In the first week BP increased, reaching the same level (121±2 mm Hg, n=12) as in NT mice (122±4 mm Hg, n=12). However, in the second week, BP decreased, achieving nonhypertensive levels (109.7±4.1 mm Hg, n=12) by the end of the Ang II infusion, although still higher than in saline-infused SA mice (93.3±3.7 mm Hg, n=5). To confirm the ability of hACE2 expression to reduce Ang II–mediated responses, we assessed the drinking behavior during the 2-week Ang II infusion. Baseline water intake was not significantly different (*P>0.05) between genotypes (Figure 2B). Ang II infusion resulted in a dramatic increase in daily water intake in NT mice by the end of the infusion protocol. Importantly, ACE2 overexpression prevented the enhanced water intake in SA compared to NT mice (4.9±0.4, n=9 versus 9.5±1.5 mL/d, n=11; *P<0.05) (Figure 2B). However, it remained higher in Ang II–infused SA compared to saline-infused mice (4.9±0.4, n=9 versus 2.6±0.2 mL/d, n=4; *P<0.01). Taken together, these data suggest that Ang II pressor and drinking responses are dramatically reduced but not totally prevented by neuronal ACE2 overexpression.

To address the participation of Ang-(1-7) in the prevention of hypertension in this model, we used D-Ala7-Ang-(1-7) infusion, to chronically block the Ang-(1-7) receptor while infusing mice with Ang II. Interestingly, the Ang-(1-7) receptor antagonist totally reversed the antihypertensive effect of ACE2 overexpression, therefore facilitating the development of hypertension (145±3 mm Hg, n=6) in SA mice (Figure 2A). D-Ala7-Ang-(1-7) alone did not alter BP. Moreover, the Ang-(1-7) receptor antagonist failed to reverse the Ang II–mediated increase in water intake in SA mice (Figure 2B). Altogether, our data suggest that Ang-(1-7) mediates the reduction of BP in SA transgenic mice, although this peptide has no effect on BP in normotensive mice and does not trigger water intake.

**Brain-Targeted ACE2 Overexpression Reinforces Spontaneous Baroreflex Sensitivity and Parasympathetic Tone**

To dissect the physiological mechanisms involved in BP regulation in SA mice, spontaneous baroreflex sensitivity (SBRS) was assessed. Baseline (saline-infused mice) SBRS was similar between SA (2.7±0.3 ms/mm Hg, n=8) and NT littermates (2.6±0.3 ms/mm Hg, n=6; *P>0.05) (Figure 2C). After 1 week of Ang II infusion, SBRS was already significantly reduced in NT mice (1.6±0.2 ms/mm Hg, n=11 *P<0.05) and remained low after 2-week infusion (1.8±0.2 ms/mm Hg, n=11; *P<0.05). In SA mice, SBRS shows a slight but nonsignificant reduction at 1 week (2.0±0.3 ms/mm Hg, n=10; *P>0.05) and remained normal until the end of the protocol (2.5±0.2 ms/mm Hg, n=14). In addition, as another mechanism controlling BP, autonomic function was assessed using classic pharmacological blockers of sympathetic and/or parasympathetic drive. Baseline parasympathetic and sympathetic tones were not significantly different between SA (changes in heart rate, ΔHR [bpm]: +206±11, n=15 and −117±18, n=9) and NT (ΔHR [bpm]: +219±9, n=7 and −93±34, n=6; *P>0.05) mice, respectively (Figure 2D and 2E). Ang II infusion significantly reduced the parasympathetic tone in NT (ΔHR: +145±6 bpm, n=6; *P<0.001) but not in SA (ΔHR: 186±13, n=6; *P>0.05) mice (Figure 2D). However, sympathetic tone was similarly increased in both genotypes following Ang II infusion (NT: −168±16, n=11; SA: −209±30 bpm, n=13) (Figure 2E). Ganglionic blockade reduced HR to the same extent in both genotypes in mice infused with saline (NT: 393±37, n=6; SA: 396±25 bpm, n=9) and Ang II (NT: 333±17, n=13; SA: 363±16 bpm, n=13), suggesting that intrinsic heart rate was not affected by ACE2 overexpression. Taken together, these data suggest that ACE2 overexpression in SA mice prevents the Ang II–mediated decreases in both SBRS and parasympathetic tone, without significantly affecting sympathetic outflow.

**ACE2 and Angiotensin Receptors Expression in the Brainstem**

We previously showed that ACE2-mediated reduction of the pressor response to Ang II is associated with down-
regulation of AT1 receptor expression in the SFO. To further elucidate the mechanisms involved in the reduction of neurogenic hypertension in SA mice, we examined Ang receptors expression in the brainstem where Ang II levels appear to be reduced in SA mice (Table 1). ACE2 overexpression led to AT1 receptors downregulation in both NTS (Table 2 and Figure 3A) and RVLM (Online Figure II). Moreover, this was associated with upregulation of AT2 and Mas receptors in SA brainstem, compared to NT mice. The resulting elevated AT2/AT1 (Figure 3B) and Mas/AT1 (Figure 3C) receptors ratios also remained higher in SA mice following Ang II infusion, suggesting that SA mice could be less responsive to Ang II stimulation. In addition, Ang-(1-7) receptor blockade resulted in a significant increase in AT1 receptors immunostaining (Table 2; *P<0.05) contributing to the reduction of the AT2/AT1 (Figure 3B) and Mas/AT1 (Figure 3C) receptors ratios. Together, these data suggest that ACE2 overexpression confers a protective effect to SA mice by modulating Ang receptors expression in the brainstem.

**ACE2 and NOS Expression**

Activation of Ang receptors has been shown to regulate NOS expression and phosphorylation thus modulating NO release in vitro and in vivo. To address the molecular mechanisms by which ACE2 prevents the development of hypertension and the Ang II–mediated decreases in SBRS and parasympathetic tone in SA mice, we investigated the regulation of AT1 receptor expression in the SFO. To further elucidate the mechanisms involved in the reduction of neurogenic hypertension in SA mice, we examined Ang receptors expression in the brainstem where Ang II levels appear to be reduced in SA mice (Table 1). ACE2 overexpression led to AT1 receptors downregulation in both NTS (Table 2 and Figure 3A) and RVLM (Online Figure II). Moreover, this was associated with upregulation of AT2 and Mas receptors in SA brainstem, compared to NT mice. The resulting elevated AT2/AT1 (Figure 3B) and Mas/AT1 (Figure 3C) receptors ratios also remained higher in SA mice following Ang II infusion, suggesting that SA mice could be less responsive to Ang II stimulation. In addition, Ang-(1-7) receptor blockade resulted in a significant increase in AT1 receptors immunostaining (Table 2; *P<0.05) contributing to the reduction of the AT2/AT1 (Figure 3B) and Mas/AT1 (Figure 3C) receptors ratios. Together, these data suggest that ACE2 overexpression confers a protective effect to SA mice by modulating Ang receptors expression in the brainstem.

**Table 2. Quantification of Brainstem AT1, AT2, and Mas Receptors Immunostaining**

<table>
<thead>
<tr>
<th></th>
<th>AT1</th>
<th>AT2</th>
<th>Mas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus of tractus solitarius</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT+saline</td>
<td>1.0±0.27</td>
<td>1.0±0.27</td>
<td>1.0±0.66</td>
</tr>
<tr>
<td>SA+saline</td>
<td>0.28±0.11*</td>
<td>2.9±0.29*</td>
<td>3.88±0.88*</td>
</tr>
<tr>
<td>NT+Ang II</td>
<td>0.57±0.29</td>
<td>0.95±0.40</td>
<td>0.80±0.11</td>
</tr>
<tr>
<td>SA+Ang II</td>
<td>0.11±0.03</td>
<td>0.77±0.14†</td>
<td>0.71±0.03</td>
</tr>
<tr>
<td>SA+Ang II+D-Ala7-Ang-(1-7)</td>
<td>8.96±0.83‡</td>
<td>1.98±0.22</td>
<td>0.81±0.14</td>
</tr>
<tr>
<td>Rostral ventrolateral medulla</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT+saline</td>
<td>1.0±0.09</td>
<td>1.0±0.24</td>
<td>1.0±0.20</td>
</tr>
<tr>
<td>SA+saline</td>
<td>1.53±0.21</td>
<td>3.2±0.24*</td>
<td>4.05±0.34*</td>
</tr>
<tr>
<td>NT+Ang II</td>
<td>1.39±0.31</td>
<td>1.12±0.13</td>
<td>1.13±0.19</td>
</tr>
<tr>
<td>SA+Ang II</td>
<td>0.92±0.15</td>
<td>0.78±0.16†</td>
<td>1.09±0.07</td>
</tr>
<tr>
<td>SA+Ang II+D-Ala7-Ang-(1-7)</td>
<td>3.57±0.82‡</td>
<td>1.43±0.26</td>
<td>0.69±0.07†</td>
</tr>
</tbody>
</table>

Data represent the relative receptor density normalized to NT+saline. Values are expressed as means±SEM. *P<0.05 vs NT for the same treatment; †P<0.05 vs saline for the same genotype; ‡P<0.05 vs SA+Ang II.
consequences of ACE2 overexpression on NOS expression and phosphorylation.

Using immunohistochemistry, total eNOS, Ser1177-phosphorylated eNOS, Thr495-phosphorylated eNOS, and nNOS expression were assessed in mouse brainstem. At baseline, ACE2 overexpression was associated with significant \((P<0.05)\) upregulation of nNOS and total eNOS in the brainstem (Figure 4A through 4C and Online Figure III). Moreover, phosphorylated eNOS-Ser1177/Thr495 ratio, an index of phosphorylation versus dephosphorylation, was significantly elevated in the NTS (Figure 4A and 4D; \(P<0.05\)). Ang II infusion dramatically reduced NOS expression in the brainstem but it remained significantly higher in SA mice than in NT mice (Figure 4B; \(P<0.05\)). Interestingly, Ang II plus D-Ala7-Ang-(1-7) dramatically decreased the phosphorylated eNOS-Ser1177/Thr495 ratio (Figure 4D; \(P<0.05\)) in SA mice, suggesting that eNOS phosphorylation is regulated by Ang-(1-7) in our model. Altogether, these data suggest that ACE2 exerts its modulatory effects partly through upregulation of NOS expression and phosphorylation, thus preventing the development of neurogenic hypertension.

ACE2 Overexpression Increases Brain NO Levels

NO is known to be released following stimulation of AT2 or Mas receptors in the brain \(^{18}\) and the periphery. \(^{19}\) To confirm the pivotal role of NO in our model, we used a free radical analyzer to directly measure NO release in the cerebrospinal fluid. Following calibration of the NO probe (Figure 5A), baseline NO release was shown to be enhanced in SA mice (3.57±0.02 versus 2.95±0.11 \(\mu\)mol/L; \(P<0.05\)) compared to controls (Figure 5B). Ang II infusion resulted in a \(\approx50\%\) reduction in NO release (1.82±0.22 \(\mu\)mol/L), whereas only \(\approx20\%\) was depleted in transgenic mice (2.68±0.19 \(\mu\)mol/L; Figure 5B). However, blockade of the Ang-(1-7) receptor in Ang II–infused SA mice further reduced NO levels to the same extent (1.75±0.08 \(\mu\)mol/L) as in Ang II–infused NT mice (Figure 5B). These data suggest that ACE2 overexpression reinforces Ang-(1-7)–mediated NO release in the brain, which might contribute to the reversal of neurogenic hypertension.

Discussion

In addition to the systemic RAS, local systems are present in various tissues and have important physiological and patho-
physiological roles. All RAS components have been identified in the brain and play important functions in cardiovascular diseases and BP regulation. As a new member of the RAS, ACE2 has been suggested to counterbalance the effects of the ACE/Ang II/AT1 receptor axis and to play a pivotal role in the maintenance of cardiovascular function. However, although evidence for a central role of ACE2 have emerged, further understanding of its importance depends on the availability of tools to manipulate its expression in the brain. As important means to investigate gene function, transgenic models have been used in the last 2 decades and have proved their value in dissecting the brain RAS. Here, we describe the characterization of a new transgenic mouse model, with brain-targeted expression of hACE2 under the control of a neuron-specific promoter. More importantly, we show evidence that brain-targeted ACE2 overexpression reverses the development of neurogenic hypertension, possibly through regulation of Ang receptors, upregulation of NOS expression and enhanced NO release in the brain.

Several studies have focused on the beneficial effects of peripheral ACE2 in the regulation of cardiovascular function, showing an association between reduction of BP and increased ACE2 in heart and kidney of SHR, as well as the beneficial effects of ACE inhibitors and AT1 receptor blockers in increasing cardiac ACE2. Further evidence of the protective role of ACE2 against cardiac hypertrophy and fibrosis, but also in reducing BP and improving vascular function, have recently emerged.

In the CNS, virus-mediated gene delivery and pharmacological studies support a role for ACE2 in BP regulation and baroreflex function. However, although these studies suggest a pivotal role for ACE2 in the SFO, NTS, and RVLM, the technical difficulties inherent to virus-mediated expression, including short duration, and the restricted availability of pharmacological agents affecting ACE2, have limited the investigation of the potential benefits of this enzyme in preventing diseases associated with the hyperactive RAS.

To clarify and further dissect the functional role of ACE2 in BP regulation and its potential for gene therapy, we engineered a new transgenic mouse model with chronic expression of hACE2 targeted to neurons in the CNS. Although SA transgenic mice harbor normal resting hemodynamic, autonomic, and baroreflex functions, the altered balances between receptors and Ang peptides levels are evidence that, when overexpressed, ACE2 can modulate the RAS, supporting the idea of a compensatory function in specific conditions. Our data show that neuron-targeted ACE2 overexpression reverses the effects of chronic administration of Ang II, thus preventing both hypertension and enhanced drinking behavior in the Ang II “slow pressor” model. In this model, infusion of a low concentration of Ang II is most effective at reaching the brain, via the blood brain barrier-deficient circumventricular organs (eg, SFO and area postrema), and acting on nuclei controlling BP rather than...
directly affecting peripheral vasculature, therefore leading to neurogenic hypertension via increased sympathetic outflow.23 However, it is important to note that like in other Ang II–mediated hypertension,24 a peripheral component (first week of infusion) exists in the slow pressor model and that this early rise in BP is not affected by ACE2 overexpression in the brain.

Although inhibition of the pressor response to acute Ang II essentially involved ACE2-mediated Ang II hydrolysis and AT1 receptors downregulation, further reducing Ang II downstream signaling,5 the reversal of neurogenic hypertension in SA mice emphasizes an important role for Ang-(1-7). Indeed, AT1 receptor expression patterns were different in SA mice, while unchanged in RVLM. Moreover, AT1 receptors were downregulated in SA mice, leading to reduced Ang II signaling. Indeed, the Ang II–induced drinking response was significantly blunted in SA mice and could not be restored to the level observed in NT mice following Ang-(1-7) receptor blockade. As first shown by Fitzsimons,34 this suggests that Ang-(1-7) is not involved in water intake, and that the impaired drinking response is attributable to a reduction in Ang II levels and downstream consistent with previous data showing that blockade of endogenous Ang-(1-7) in the paraventricular nucleus, reduced renal sympathetic tone thus suggesting that Ang-(1-7) may also participate in the maintenance of sympathetic outflow.26 Alternatively, this absence of reduction in sympathetic drive could be attributable to the lack of sensitivity of our pharmacological approach because urinary norepinephrine levels are reduced in Ang II–infused SA mice (Y.F., unpublished data).

Brain AT1 receptors are well known for promoting enhanced sympathetic tone leading to the development of hypertension and chronic heart failure.27 Studies have shown that Mas and AT2 receptors oppose AT1 receptors both in the brain and the periphery.10,28,29 We previously showed that acute ACE2 overexpression in the brain resulted in AT1 receptors downregulation.5 Here, we confirm and extend this observation by also noting that both Mas and AT2 receptors were upregulated in the presence of overexpressed ACE2. Moreover, the AT1 receptor expression patterns were different in NTS and RVLM. In NTS, AT1 receptors were downregulated in SA mice, while unchanged in RVLM. Overall, the balance between AT2/AT1 and Mas/AT1 was dramatically altered, shifting the equilibrium from increased sympathetic outflow and neurogenic hypertension (AT1 activation) toward enhanced vagal tone and BP normalization. Similar findings were recently reported by Ferreira et al, showing that the ACE2 activator XNT increased the Mas/AT1 receptors ratio in pulmonary hypertension.10 An imbalance between AT1 and AT2 in the brainstem has also been reported to affect sympathetic tone and overexpression of AT2 receptors in the RVLM was shown to promote sympathoinhibition.51,52 Interestingly, we observed that both Ang II and the Ang-(1-7) receptor blocker reverse the increased AT2/AT1 and Mas/AT1 ratios. Whereas increased AT1 receptors level, and therefore a reduction of the ratios, might be explained by a positive feedback of Ang II on its main receptor, the mechanism(s) by which d-Ala7-Ang-(1-7) may affect the receptors balance is less clear. Clark et al reported downregulation of AT1 receptors by Ang-(1-7) through a cyclooxygenase-dependent pathway in the kidney, indicating the participation of Ang-(1-7) and probably NO in these regulations.33 Our data suggest that the regulation of these receptors is probably involving several mechanisms and our observation are the sum of positive and negative feedbacks. Clearly, more investigation is needed to elucidate these receptors interactions.

The present data suggest ACE2 overexpression in the brain resulted in AT1 receptors downregulation in SA mice, leading to reduced Ang II signaling. Indeed, the Ang II–induced drinking response was significantly blunted in SA mice and could not be restored to the level observed in NT mice following Ang-(1-7) receptor blockade. As first shown by Fitzsimons,34 this suggests that Ang-(1-7) is not involved in water intake, and that the impaired drinking response is attributable to a reduction in Ang II levels and downstream
signaling. These are further evidence of the dichotomy between BP and body fluid regulation in the CNS.

In addition to modulating baroreflex and autonomic function, Ang-(1-7) has been reported to increase NO release in the brain where this neuromediator can modulate the regulation of baroreflex, autonomic function and BP. Ang-(1-7) has been shown to activate eNOS via an Akt-dependent pathway in vitro and to produce hypotensive effects by generating NO via activation of nNOS in the CVLM.

Interestingly, we observed that overexpression of ACE2 in the brain of SA mice is associated with increased eNOS and nNOS levels in key nuclei involved in the central regulation of BP, like the NTS and RVLM. More importantly, ACE2 overexpression prevented the Ang II–induced decrease in NOS expression in the NTS, suggesting that the protective effects of ACE2 could be linked to increase NO availability in this area.

Neurons in the NTS receive and integrate inputs from the periphery and higher brain structures and subsequently influence the activity of the principal brain nuclei governing efferent parasympathetic and sympathetic drive. Consistent with our observations, nNOS expression was reduced in the brainstem of a myocardial infarction mouse model, whereas adenovirus-mediated eNOS expression resulted in robust NO production and attenuation of the enhanced sympathetic nerve activity. However, although eNOS in the RVLM has been reported to improve baroreflex function, it has opposite effects in the NTS. Therefore, enhanced expression of eNOS in the NTS of SA mice may not be responsible for the improved baroreflex gain in these animals, but this could be achieved by increased nNOS in the ventral medulla. Direct measurement in SA mice reveals increased NO levels in the cerebrospinal fluid, suggesting that the overall improvements in autonomic and baroreflex function, observed in this model, could originate from various brain regions. Again, more work is needed to dissect this mechanism.

One concern with SA mice is the widespread high level expression of ACE2 throughout the brain. Although we previously showed that endogenous ACE2 expression is also prevalent in the CNS, it is possible that SA mice express the enzyme in places where it is not normally. However, for the enzyme to be active in such places it would need to have access to its substrate and the target Ang receptor, making it unlikely for the activation of nonphysiological pathways.

In summary, we have generated a new transgenic mouse model with overexpression of ACE2 in the brain. Whereas these mice have normal cardiovascular parameters, they exhibit altered NOS and Ang receptors expression at baseline. Importantly, brain-targeted ACE2 overexpression reverses neurogenic hypertension, partially by preventing the decrease in both SBRS and parasympathetic tone. Ang-(1-7) plays a pivotal role in this reversal, promoting NOS activation and leading to enhanced NO release in the CNS. In addition to generating a new transgenic mouse model that will be critical to further dissect the role of ACE2 in the brain, we provide evidence of the mechanism of action by which ACE2 prevents the development of neurogenic hypertension, therefore supporting its beneficial role as a potential drug target for the treatment of hypertension.

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Disclosures
None.

References


Brain-Selective Overexpression of Human Angiotensin-Converting Enzyme Type 2 Attenuates Neurogenic Hypertension
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Material and Methods

Generation of transgenic mice. 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 (NCBI accession number: AF291820) was constructed (Figure 1A). The transgene segment was obtained by digestion with XhoI and SpeI, separated by agarose gel electrophoresis, and recovered using the Qia-Quick gel purification kit (Qiagen Technologies, Hilden, Germany). Transgenic mice were generated by microinjection into fertilized C57BL/6JxSJL/J (B6SJLF2) mouse embryos at The University of Iowa Transgenic Facilities, positive founders were backcrossed to C57BL/6J beyond 7 generations and bred for study as described previously. All mice were fed standard mouse chow and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Iowa and Louisiana State University Health Science Center.

RNA isolation and reverse transcription PCR. Total RNA was isolated from various tissues from both transgenic and NT mice, using a standard RNA extraction procedure (TRIZOL, Invitrogen, Carlsbad, CA) as described. Isolated RNA was treated with RNase-free DNase I (Qiagen Technologies, Hilden, Germany) to remove any contaminating genomic DNA. Total RNA was quantified using a Biophotometer (Beckman counter DU 640). Complementary DNA synthesis was carried out using AMV Reverse Transcriptase and Random primers (Biorad laboratories, Hercules, CA). This cDNA was used as template for PCR analysis. RNA in the absence of reverse transcriptase was used as a negative control. Specific primers for human ACE2 (Fwd: 5'-TGA AAA ATG AGA TGG CAA GAG-3'; Rev: CAT TTC ATT GTC GTT CCA TTC ATA-3'; amplicon length: 1364 bp) and internal control mouse β-actin (Fwd: 5'-TGT GAT GGT GCC AGA TCT TCT CCA TGT-3'; amplicon length: 140 bp) were designed using PrimerQuest Software (IDT, Coralville, IA). The PCR was performed with 1 μl samples in a total volume of 20 μl consisting of 5U/µl Taq DNA polymerase (Qiagen Technologies, Hilden, Germany), 10 μM for each dNTP, 25 mmol/L MgCl₂, PCR buffer, 20 μmol/L each of forward and reverse primers. Cycling conditions were 95 °C for 5 min, and then 35 cycles consisting of 45 sec at 95 °C, 45 sec at 56 °C and 1 min at 72 °C, plus an additional extension at 72 °C for 5 min.

Western blotting. Tissue from brain cortex, hypothalamus and brainstem were collected separately and homogenized with a glass pestle in the cell lysis buffer (in mmol/L: HEPES: 10, NaCl: 150, MgCl₂: 5, EGTA: 1, 0.02% (w/v) NaN₃, pH 7.4) containing a protease inhibitors cocktail (Sigma, St Louis, MO). The lysate was centrifuged at 10,000 rpm, 4 °C for 15 min and the supernatant transferred to a clean tube. Proteins concentration was measured using a BCA assay kit (Pearce, Rockford, IL). Cells lysates (10 μg) were mixed with SDS-PAGE sample buffer (0.125 mol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue), heated at 100 °C for 5 min and loaded onto a 15% SDS–polyacrylamide gel for electrophoresis. Proteins were transferred to nitrocellulose membrane at 200 mA for 1 hr by semi-dry blot (Fisher Scientific, Houston, TX). Membranes were blocked with 5% non-fat milk in PBS-T (1.47 mmol/L NaH₂PO₄, 8.09 mmol/L Na₂HPO₄, 145 mmol/L NaCl, 0.05% (v/v) Tween-20, 0.01% (w/v) thimerosal, pH 7.4) for 2 hr at room temperature and incubated with a goat-anti-human ACE2 antibody (AF933, R&D Systems, Minneapolis, MN) in 1:1000 dilution for 1 hr at room temperature. Membranes were washed with PBST 4 times for 5 min then incubated with donkey anti-goat IgG-HRP (ab6885, abCAM, Cambridge, MA, 1:5000) and goat anti-mouse beta-actin antibody (ab8229, abCAM, 1:5000) for 45 min at room temperature, as described. Specific bands were detected by chemiluminescence according to the manufacturer’s instructions (ECL®, Perkin Elmer, Boston, MA).

ACE2 activity. Tissue from brain cortex, hypothalamus and brainstem were collected separately and homogenized with a glass pestle in 1 mL ACE2 activity reaction buffer (in mmol/L: NaCl: 1000, Tris: 75, ZnCl₂: 0.5, pH 7.5) and centrifuged at 20,800 x g for 10 min and the supernatant transferred to a clean tube. Proteins concentration was measured using a BCA assay kit (Pearce, Rockford, IL). ACE2 activity measurement was carried out in the presence of captopril to eliminate any contribution by endogenous

Supplement Material
ACE and based on the use of the Fluorogenic Peptide Substrate VI (FPSVI, 7Mca-Y-V-A-D-A-P-K(Dnp)-OH)(R&D systems, Minneapolis, MN) as described previously. This substrate contains a C-terminal dinitrophenyl moiety that quenches the inherent fluorescence of the 7-methoxycoumarin group by resonance energy transfer. ACE2 removes the C-terminal dinitrophenyl moiety in the FPSVI thus increasing fluorescence emission at 405 nm under excitation at 320 nm. Tissue lysates containing 100 μg of protein, were incubated with FPSVI (100 μmol/L) and captopril (10 μmol/L) in reaction buffer (100 μL) at room temperature. Non specific enzyme activity was measured by including DX600 (1 μmol/L), a specific ACE2 inhibitor, (Phoenix Pharmaceutical, Belmont, CA). Fluorescence emission was monitored using a SpectraMax M2 Fluorescence Reader (Molecular Devices, Sunnyvale, CA). The noise to signal ratio for enzyme activity in the absence of substrate or cell extract was <5%. Specific ACE2 activity was calculated by subtracting the total activity in the presence of 10 μmol/L captopril from the activity in the presence of both 10 μmol/L captopril and 1 μmol/L DX600. Data (arbitrary fluorescence units, AFU) are presented as amounts of substrate FPSVI converted to product per minute and are normalized for total protein.

**Radioimmunoassays (RIAs) of Angiotensin II and Ang 1-7 levels.** Male syn-hACE2 and control littersmates (n=8), 8-10 weeks old, were anesthetized and infused subcutaneously for 14 days using osmotic minipumps (Alzet) containing either 1) saline, 2) Ang-II (600 ng/kg.min) 3) the Ang-(1-7) receptor blocker D-Ala²-Ang-(1-7) (600 ng/kg.min) or 4) Ang-II + D-Ala²-Ang-(1-7). At the end of the 2-week infusion, blood was collected following decapitation, in the presence of 0.44 mmol/L 1,10-phenanthroline monohydrate (Catalog number P-1294, Sigma, St. Louis MO.), 0.12 mmol/L pepstatin (Peninsula Labs, Belmont CA), 1 mmol/L sodium p-hydroxymercuribenzoate (Catalog number H 0642, Sigma, St. Louis MO), 15% EDTA, and 0.01 mmol/L WFML-1, a rat renin inhibitor (AnaSpec Inc, San Jose, CA). Brain regions were quickly dissected over ice and frozen in liquid nitrogen. Blood and tissue samples were immediately shipped in dry ice to the Wake Forest University Hypertension Core Laboratory tissues for processing. Blood samples were centrifuged at 2000 rpm for 10 minutes at 4°C. Plasma was transferred into a pre-chilled centrifuge tube and spun at 2000 rpm for 10 more minutes at 4°C. Brain regions were homogenized in acid/ethanol [80% vol/vol 0.1 mol/L HCl] containing a cocktail of protease inhibitors including 0.44 mmol/L 1,10-phenanthroline monohydrate (Sigma, St. Louis MO.), 0.12 mmol/L pepstatin (Peninsula Labs, Belmont CA), 1 mmol/L sodium p-hydroxymercuribenzoate (Sigma, St. Louis MO), 15% EDTA, and 0.01 mmol/L WFML-1, a rat renin inhibitor (AnaSpec Inc, San Jose, CA), centrifuged at 12000 rpm for 20 minutes at 4°C and stored overnight at 4°C. Samples were re-centrifuged at 12000 rpm for 20 min at 4°C, the supernatant was removed, added to 1% HFBA and refrigerated overnight at -20°C. The supernatant was extracted using Sep-Pak columns activated with 5 ml wash of a mixture of n-heptfluorobutryc acid (HFBA): methanol (0.1%:80%) and sequential washes of 0.1% HFBA. After the sample was applied to the column, it was washed with 0.1% HFBA and followed with a water wash. The samples were eluted with 3.3 ml washes of a mixture of acid methanol (0.1%:80%), reconstituted and split for the two RIAs. For Ang II, samples were reconstituted in assay buffer and for Ang-(1-7), a TRIS buffer with 0.1% BSA was used. Recoveries of radiolabeled angiotensin added to the sample and followed through the homogenization and extraction were 68% (n=23). Samples were corrected for recoveries. Ang II was measured using a radioimmunoassay kit (Alpco, Windham, NH) and Ang-(1-7) was measured using the antibody previously described. The minimum detectable levels of the assays were 0.8 fmol/ml and 2.8 fmol/ml for Ang II, and Ang-(1-7), respectively. Values at or below the minimum detectable level (MDL) of the assay were arbitrarily assigned that value for statistical analysis. The intra-assay and inter-assay coefficient of variation was 12 and 22% for Ang II, and 8% and 20% for Ang-(1-7).

**Immunohistochemistry.** Detection of human ACE2 and NOS was performed as described previously. Mice were deeply anesthetized with Nembutal and perfused transcardially with PBS (0.1 mmol/L, pH 7.4) for 2 min followed by 4% paraformaldehyde in PBS (0.1 mmol/L, pH 7.4) for 10 min. The brain was then removed, post fixed for 1 hr in 4% paraformaldehyde in PBS (0.1 mmol/L, pH 7.4) and then placed in 20%
Nitric oxide (NO) measurement. Male syn-hACE2 and NT mice (n=8/group), 8-10 weeks old, were anesthetized and infused subcutaneously for 14 days using osmotic minipumps (Alzet) containing either 1) saline, 2) Ang-II (600 ng/kg.min) 3) the Ang-(1-7) receptor blocker D-Ala^7^-Ang-(1-7) (600 ng/kg.min) or 4) Ang-II + D-Ala^7^-Ang-(1-7). At the end of the 2-week infusion, mice were anesthetized and NO levels were measured using a NO detection probe (NS-ISO-NOPF100-1MM WPI Inc, FL) as described by the manufacturer. The NO probe was polarized in 0.3 mmol/L CuCl₂ at 37 °C for about 2 hours until stabilization of the current. A standard curve was generated by adding serial concentrations of S-nitroso-N-acetyl-L-1-penicillamine (SNAP). The NO probe was then lowered into the mouse lateral ventricle using previously reported coordinates^5 and the current was measured for at least 5 minutes post-stabilization. Conversion to nitric oxide concentrations (µ mmol/L) was performed according to the standard curve.

Physiological recordings. Male syn-hACE2 and control littermates (n=12), 8-10 weeks old, were anesthetized and instrumented with a radiotelemetry probe, as described. ^3,6 After 14 days recovery, baseline BP was recorded for 4 days. Mice were then infused subcutaneously for 14 days using osmotic minipumps (Alzet) containing either 1) saline, 2) Ang-II (600 ng/kg.min) 3) the Ang-(1-7) receptor blocker D-Ala^7^-Ang-(1-7) (600 ng/kg.min) or 4) Ang-II + D-Ala^7^-Ang-(1-7). Water intake was recorded daily. Autonomic function was assessed in conscious freely moving mice, before and at the end of the infusion protocol, by using a standard pharmacological method involving random ip injection of a β-blocker (propranolol, 4 mg/kg), a muscarinic receptor blocker (atropine, 1 mg/kg) and a ganglionic blocker (hexamethonium, 10 mg/kg). ^7 Each injection was separated by at least a 3-hour recovery period. Changes in HR were calculated following administration of the antagonists. Spontaneous baroreflex sensitivity was calculated using the sequence method as used previously. ^5

Statistical Analysis. Data are expressed as mean ±SEM. Data were analyzed by Student’s t test or two-way ANOVA (Bonferroni post hoc tests to compare replicate means) when appropriate. Statistical comparisons were performed using Prism5 (GraphPad Software, San Diego, CA). Differences were considered statistically significant at P<0.05.

References


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**Online Table I.** Baseline hemodynamic parameters and activity.

<table>
<thead>
<tr>
<th></th>
<th>MAP (mmHg)</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>PP (mmHg)</th>
<th>HR (bpm)</th>
<th>Activity (AU)</th>
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<td><strong>24 hr (6 PM–6 PM)</strong></td>
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<td><strong>Control</strong></td>
<td>97.2±2.2</td>
<td>106.9±3.1</td>
<td>83.2±2.0</td>
<td>23.6±3.5</td>
<td>527.7±8.4</td>
<td>6.3±0.8</td>
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<td><strong>Syn-hACE2</strong></td>
<td>101.8±2.0</td>
<td>115.1±2.3</td>
<td>87.6±1.9</td>
<td>27.4±2.0</td>
<td>556.5±8.6</td>
<td>5.1±0.5</td>
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<tr>
<td><strong>Day (6 AM–6 PM)</strong></td>
<td></td>
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<tr>
<td><strong>Control</strong></td>
<td>95.2±4.7</td>
<td>104.8±3.5</td>
<td>81.3±2.3</td>
<td>23.4±3.6</td>
<td>515.5±7.8</td>
<td>5.2±0.8</td>
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<tr>
<td><strong>Syn-hACE2</strong></td>
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<td>114.4±2.4</td>
<td>86.8±1.9</td>
<td>27.6±2.0</td>
<td>550.4±8.9</td>
<td>4.4±0.4</td>
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<tr>
<td><strong>Night (6 PM–6 AM)</strong></td>
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<tr>
<td><strong>Control</strong></td>
<td>99.1±7.2</td>
<td>109.4±2.7</td>
<td>85.4±1.8</td>
<td>23.9±3.4</td>
<td>535.4±9.6</td>
<td>7.6±0.8</td>
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<tr>
<td><strong>Syn-hACE2</strong></td>
<td>102.9±6.2</td>
<td>115.6±2.2</td>
<td>88.4±1.9</td>
<td>27.1±2.0</td>
<td>564.6±8.4</td>
<td>5.9±0.7</td>
</tr>
</tbody>
</table>

Data represent the average of the daily 24hr means in each animal. Values are mean ±SEM. Abbreviations: MAP, mean arterial pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; HR, heart rate; bpm, beats/min; AU, arbitrary units.
Online Figure I. Expression of hACE2 in transgenic mice. Representative immunohistochemistry pictures showing hACE2 expression in NT (left) and SA (right) mice. Low reactivity was observed in NT mice (A, C, E, G, I, K), while Widespread expression was detected throughout the brain of SA including in cardiovascular: 10N (B), AP (D), MnPO (H) and PVN (L), and non-cardiovascular regions: cortex (F) and OVLT (J). Abbreviations: 10N, dorsal motor nucleus of the vagus nerve; AP, area postrema; MnPO, median preoptic area; OVLT, organum vasculosum of the lamina terminalis; PVN, paraventricular nucleus.
Online Figure II. Angiotensin AT₁, AT₂ and Mas receptors expression in the RVLM. (A) Representative immunohistochemistry pictures for AT₁, AT₂ and Mas receptors (n=3/group). In the RVLM, AT₂/AT₁ (B) and Mas/AT₁ (C) receptors ratios were significantly (P<0.05) increased in SA compared to NT mice. Ang II infusion reduced the ratios in SA mice to similar levels than in NT mice. They were further reduced (P<0.05) after Ang-II+D-ala⁷-Ang-(1-7). *P<0.05 vs. NT; †P<0.05 vs. baseline and §P<0.05 vs. SA + Ang II.
Online Figure III. NOS expression in the RVLM. (A) Representative immunohistochemistry images for nNOS, total eNOS, phos-eNOS Ser\textsuperscript{1177} and phos-eNOS Thr\textsuperscript{495} (n=3/group). Baseline, nNOS (B) and total eNOS (C) were significantly (P<0.05) increased in SA compared to NT mice. Following Ang II infusion, nNOS and total eNOS expression were similarly decreased in SA mice and NT mice while phos-eNOS Ser\textsuperscript{1177}/Thr\textsuperscript{495} ratio (D), an index of phosphorylation vs. dephosphorylation, was significantly increased. Ang-II+D-ala\textsuperscript{7}-Ang-(1-7) significantly increased the nNOS (B) and eNOS (C) expression in SA mice (P<0.05) while the phos-eNOS Ser\textsuperscript{1177}/Thr\textsuperscript{495} ratio was dramatically (P<0.05) reduced after Ang-II+D-ala\textsuperscript{7}-Ang-(1-7). Statistical significance: \textasteriskcentered*P<0.05 vs. NT; \textdagger P<0.05 vs. baseline and \textdaggerdbl P<0.05 vs. SA + Ang II.