Angiotensin-Converting Enzyme 2 Overexpression in the Subfornical Organ Prevents the Angiotensin II–Mediated Pressor and Drinking Responses and Is Associated With Angiotensin II Type 1 Receptor Downregulation

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Abstract—We recently reported the presence of angiotensin-converting enzyme (ACE)2 in brain regions controlling cardiovascular function; however, the role of ACE2 in blood pressure regulation remains unclear because of the lack of specific tools to investigate its function. We hypothesized that ACE2 could play a pivotal role in the central regulation of cardiovascular function by regulating other renin–angiotensin system components. To test this hypothesis, we generated an adenovirus expressing the human ACE2 cDNA upstream of an enhanced green fluorescent protein (eGFP) reporter gene (Ad-hACE2-eGFP). In vitro characterization shows that neuronal cells infected with Ad-hACE2-eGFP (10 to 100 multiplicities of infection), but not Ad-eGFP (100 multiplicities of infection), exhibit dose-dependent ACE2 expression and activity. In addition, an active secreted form was detected in the conditioned medium. In vivo, Ad-hACE2-eGFP infection (2 × 10⁶ plaque-forming units intracerebroventricularly) produced time-dependent expression and activity (with a peak at 7 days) in the mouse subfornical organ. More importantly, 7 days after virus infection, the pressor response to angiotensin (Ang) II (200 pmol intracerebroventricularly) was significantly reduced in Ad-hACE2-eGFP–treated mice compared with controls. Furthermore, subfornical organ–targeted ACE2 overexpression dramatically reduced the Ang II–mediated drinking response. Interestingly, ACE2 overexpression was associated with downregulation of the Ang II type 1 receptor expression both in vitro and in vivo. These data suggest that ACE2 overexpression in the subfornical organ impairs Ang II–mediated pressor and drinking responses at least by inhibiting the Ang II type 1 receptor expression. Taken together, our results show that ACE2 plays a pivotal role in the central regulation of blood pressure and volume homeostasis, offering a new target for the treatment of hypertension and other cardiovascular diseases. (Circ Res. 2008;102:729-736.)

Key Words: adenovirus ■ carboxypeptidase ■ brain ■ blood pressure ■ gene therapy

The renin–angiotensin system (RAS) is well known for its effects on the cardiovascular system and fluid homeostasis. Classically, these effects were thought to result primarily from the systemic production of angiotensin (Ang) II.1 Circulating Ang II stimulates Ang II type 1 (AT₁) receptors present in the kidney and the vasculature to produce vasoconstriction but also water and salt reabsorption. Although kidneys and liver are the major endocrine sites of renin and angiotensinogen synthesis, respectively, this view of the RAS has been challenged in the last decade because both genes have been detected in extrarenal and extrahepatic tissues.2,3 For instance, it has become clear that a local RAS is present in several tissues, for example, the heart, adipose, vasculature, and bone marrow, with similar effects to the endocrine RAS but also more specific functions depending on the individual system.1 One of these local systems, the brain RAS, has long been considered pivotal in cardiovascular regulation and important in the pathogenesis of hypertension and heart failure.2 Yet the brain RAS remains poorly understood, because of the difficulty in experimentally dissecting the brain RAS at the cellular, regional, and whole organism levels.

In the year 2000, a new member of the ACE family was identified and named Ang-converting enzyme (ACE)2.4 This carboxypeptidase was first sequenced and cloned from human heart failure ventricle and human lymphoma cDNA libraries. These studies reported major expression of ACE2 mRNA in heart and kidneys but failed to detect it in the brain. Later studies reported ACE2 mRNA in rat medulla oblongata5 and ACE2 activity in mouse brain.6 Recently, we showed,
for the first time, the presence of ACE2 protein and mRNA in the mouse brain, including in regions involved in the central regulation of cardiovascular function. In addition to identifying ACE2 as a new member of the brain RAS, we showed that this carboxypeptidase is highly regulated by other components of this system, both in normotensive and hypertensive mice. ACE2 has been reported to degrade Ang II into the vasodilatory peptide Ang-(1-7) with an affinity 400-fold greater than for Ang I. Although central Ang-(1-7) has been shown to enhance baroreceptor reflex sensitivity and exert pressor or depressor responses depending on the targeted region, the role of ACE2 in central cardiovascular regulation remains unclear because of the lack of specific tools to investigate its function.

We hypothesized that ACE2 could play a major role in the central regulation of the autonomic nervous system by increasing Ang-(1-7) and buffering the effects of enhanced Ang II levels in cardiovascular diseases. To test this hypothesis, and selectively manipulate ACE2 expression in specific tissues or cells, we developed an adenovirus coding for human (h)ACE2. Our data demonstrate that the enhanced green fluorescent protein (eGFP) reporter gene. Our data demonstrate that Ad-hACE2-eGFP induces high levels of ACE2 mRNA, protein, and activity in neurons. More importantly, intracerebroventricular (ICV) Ad-hACE2-eGFP infection resulted in high ACE2 activity levels in the mouse subfornical organ (SFO), associated with a reduction in AT1 receptor expression and leading to significant reductions in Ang II–mediated pressor and drinking responses. Consequently, our results establish a role for ACE2 in the central regulation of cardiovascular function, offering a new target for the treatment of hypertension and other cardiovascular diseases.

Materials and Methods
An expanded Materials and Methods section is in the online data supplement, available at http://circres.ahajournals.org.

Adenovirus Generation
The Ad-hACE2-eGFP virus was developed in collaboration with the University of Iowa Gene Transfer Vector Core. Briefly, the ACE2 pcDNA3.1 vector (kind gift of Dr Curt D. Sigmund, University of Iowa, Iowa City) was digested with XbaI and Pmel to excise the 2418-bp hACE2 fragment (GenBank accession no. AF291820). This fragment was then cloned into a pacAd5 CMV IRES eGFP pA shuttle (Figure IA in the online data supplement). The resulting construct was then used to generate the hACE2-eGFP adenovirus as described.

Cell Culture and Adenovirus Infection
Neuro-2A cells (mouse neuroblastoma; American Type Culture Collection, Manassas, Va) were grown in 6-well plates at a density of 2 × 10^5 cells per well. After 24 hours, cells were incubated in a low FBS medium (2%) in the presence of Ad-hACE2-eGFP (10, 50, 100 multiplicities of infection [MOIs]) virus or Ad-eGFP control virus (100 MOIs) for 6 hours, then returned to a 10% FBS medium. On the third day after infection, cells were examined using a fluorescence microscope (Olympus, IX81), and then media and cells were collected and assayed as described below.

In Vivo Adenovirus ICV Injection
Male C57Bl/6J mice, 8 to 10 weeks old (Charles River Laboratories, Wilmington, Mass), were anesthetized and Ad-hACE2-eGFP or Ad-eGFP were injected ICV (2 × 10^7 plaque-forming units [pfu], 200 nL) using a pressure injector (PicospritzerrII). Mice were euthanized 7 days after infection. Brains were either (1) sectioned coronally in a cryostat, then processed for GFP fluorescence visualization and hACE2 or AT1 immunohistochemistry; or (2) frozen at −80°C before the SFO was dissected and used for the following assays. All procedures were approved by the Institutional Use and Care Committee at Louisiana State Health Sciences Center, New Orleans.

AT1 Receptor Binding
The AT1 receptor density was determined in enriched plasma membrane preparations from mouse SFOs (n = 20 per group) and neuro-2A cells following infection with Ad-hACE2-eGFP or Ad-eGFP. The membrane suspension (100 µg per reaction) was incubated with 100 pmol/L 125I-[Sar1,Ile8]Ang II (Perkin Elmer, specific activity: 2200 Ci/mmol) for 2 hours at room temperature. Nonspecific binding was determined in the presence of 5 µmol/L nonradioactive Ang II.

Western Blot
Cell culture media (10 µL), cell lysates (10 µg), and purified SFO membranes (5 µg) were collected separately and processed using a standard Western blot protocol against hACE2 or mouse AT1, AT2, and AT3 antibodies. Specific bands were detected by chemiluminescence according to the instructions of the manufacturer (ECL, Perkin Elmer, Boston, Mass) and quantitated by laser densitometry (FujiFilm, ImageReader version 1.2).

ACE2 Activity
Cells and culture media were collected 3 days after infection, and mouse SFOs were collected 7 days after Ad-hACE2-eGFP (n = 15) or Ad-eGFP (n = 15) (2 × 10^6 pfu ICV). ACE2 activity was measured in cell lysates, cell culture media, and SFO lysate, as described.

Immunohistochemistry
Seven days after infection with Ad-hACE2-eGFP (n = 5) or Ad-eGFP (n = 5), brains were perfused, postfixed overnight, and cryosectioned, as described. Brain sections and cell cultures were processed for hACE2 or AT1 receptor detection (1:50 dilution for 48 hours). Immunostaining was detected using fluorescence (Olympus, IX81) and bright field (Nikon Eclipse E600) microscopes.

Physiological Recordings
Male C57Bl/6J mice (n = 25) were anesthetized and instrumented with an ICV canula and a radiotelemetry probe, as described. Conscious mice were then injected ICV with Ad-hACE2-eGFP (n = 13) or Ad-eGFP (n = 12) (2 × 10^6 pfu, 200 nL). After 7 days, blood pressure (BP) was recorded at baseline and following ICV injection of Ang II (200 ng, 200 nL), carbachol (100 ng, 200 nL), or the Ang-(1-7) receptor blocker D-Ala7-Ang-(1-7) (200 fmol, 200 nL) before ICV Ang II. Water intake was monitored by recording the time spent drinking during the 15 minutes following ICV Ang II administration. From baseline recordings, spontaneous baroreceptor reflex sensitivity (SBRS) was calculated using the sequence method as described.

Statistical Analysis
Data are expressed as means ± SEM. Data were analyzed by Student’s t test or ANOVA (after Bartlett’s t test of homogeneity of variance), followed by Newman–Keuls correction for multiple comparisons between means. Statistical comparisons were performed using Prism4 (GraphPad Software, San Diego, Calif). Differences were considered statistically significant at P < 0.05.
Results

In Vitro Characterization of the Adenovirus

To overcome the lack of tools to investigate the role of the brain RAS in hypertension and particularly the involvement of ACE2 in the central regulation of BP, we developed a new adenovirus coding for the hACE2 carboxypeptidase. First, we investigated whether the adenovirus coding for the full-length hACE2 upstream of an eGFP reporter gene could be expressed properly in neuronal cells. Neuro-2A cells were infected with Ad-hACE2-eGFP or Ad-eGFP and infection efficiency was evaluated after 3 days with regard to GFP fluorescence expression, ACE2 protein and activity levels in both cell lysate and culture medium. Despite the short term exposure to the virus, highly efficient infection was achieved as evidenced by visualization of eGFP fluorescence (supplemental Figure IB). In addition, in cells infected with Ad-hACE2-eGFP, GFP fluorescence was colocalized with hACE2 expression (Figure 1A), confirming that GFP fluorescence is a reliable index of hACE2 expression. As shown previously, virus infection did not significantly affect cell viability for titers ranging from 10 to 100 MOIs (data not shown). hACE2 protein expression was dose-dependently increased in neuro-2A cells following Ad-hACE2-eGFP infection (Figure 1B and 1C). Additional experiments using an antibody targeting the hACE2 signal peptide identified hACE2 in cell lysates but not in cell culture medium (supplemental Figure IC), suggesting normal excision of the signal peptide thus permitting hACE2 release into the surrounding milieu.

To determine the functionality of the protein, ACE2 activity was measured. All Ad-hACE2-eGFP virus titers, but not Ad-eGFP, induced a significant increase in ACE2 activity in both cell lysate (532.4±80.1 versus 5.5±3.4 AFU/mg·min⁻¹ for 100 MOIs of Ad-hACE2-eGFP and Ad-eGFP, respectively; P<0.001) and culture medium (349.6±71.7 versus 42.2±7.2 AFU/mL·min⁻¹ for 100 MOIs of Ad-hACE2-eGFP and Ad-eGFP, respectively; P<0.01) (Figure 1D), confirming the ability of hACE2 to efficiently hydrolyze target peptides. Taken together, these data provide evidence that Ad-hACE2-eGFP can be used to express a functional and active hACE2 protein in neurons in an efficient and easily traceable manner.

SFO-Targeted Expression of hACE2 in Mice

To determine whether ACE2 participates in the central regulation of BP, we overexpressed the enzyme in the SFO, a brain region known for its involvement in the regulation of cardiovascular function. C57Bl/6J mice were infected ICV with the virus to determine the course of expression and activity in the brain. Figure 2A shows the localization of the ICV injection site relative to the SFO on a coronal section of the mouse brain. The viral infection efficiency was determined by using eGFP as a reporter gene.

Measurements of ACE2 activity in the whole hypothalamus at different time points showed a significant increase as early as 3 days after infection (24.9±1.4 versus 2.7±3.3 AFU/mg·min⁻¹ for the control virus; P<0.001) and achieved a maximum 7 days after infection (33.3±5.9 versus 2.7±3.3 AFU/mg·min⁻¹ for the control virus; P<0.001). Expression of hACE2 in the mouse brain correlates with the activity time course, as evidenced by cells expressing GFP as early as day 3 (data not shown) and very high fluorescence at 7 days after infection (supplemental Figure II). High hACE2 expression (visualized by GFP fluorescence) following ICV injection of Ad-hACE2-eGFP was essentially restricted to the SFO. GFP expression in the SFO, 7 days after Ad-hACE2-eGFP infection, was identified in the core and horns of the organ as well as in the surrounding tissue. Regions exhibiting weaker fluorescence included the wall of the lateral ventricle, the dorsal third ventricle, the fimbria of the hippocampus, and the ventral hippocampal commissure (data not shown).

Despite the persistence of few cells still harboring GFP fluorescence in the wall of the lateral ventricle (data not shown), ACE2 activity had returned to baseline (below detection levels) at 14 and 28 days after Ad-hACE2-eGFP infection (supplemental Figure II). The time course and pattern of expression obtained with Ad-hACE2-eGFP were identical to previous data using similar constructs. To confirm that our activity measurements in the hypothalamus and the use of eGFP as a reporter gene are representative of ACE2 activity and expression in the SFO, we performed these measurements directly in the SFO. ACE2
activity was significantly increased in the SFO (Figure 2B) in Ad-hACE2-eGFP–infected mice (353.4±23.7 AFU/mg·min⁻¹) compared with Ad-eGFP–treated mice (2.4±1.7 AFU/mg·min⁻¹) 7 days after infection. Similarly, hACE2 protein expression was detected only in the SFO of Ad-hACE2-eGFP–infected mice (Figure 2C and 2D). As observed previously with eGFP, immunohistochemistry for hACE2 shows SFO-restricted expression of the carboxypeptidase (Figure 2E) 7 days after ICV injection of Ad-hACE2-eGFP. In Ad-eGFP–infected mice, hACE2 expression was undetectable (Figure 2F). Higher magnification showed eGFP and hACE2 expression in both neurons and glia, consistent with the adenovirus lack of cell tropism. However, hACE2 expression to glial cells was extremely weak compared with neurons (supplemental Figure III). Moreover, consistent with our previous observations, hACE2 cellular expression was localized to the cytoplasm and the cell membrane.⁴,⁷

Taken together, these data indicate that Ad-hACE2-eGFP induces a functional hACE2 expression in the brain for at least 7 days, allowing its use to investigate the role of ACE2 in regulating central cardiovascular function.

**ACE2 Expression and Ang II–Mediated Pressor and Drinking Responses**

To assess the ability of ACE2 to counter the effects of Ang II in the SFO, conscious freely moving mice previously infected with Ad-hACE2-eGFP (7 days) were injected ICV with Ang II (200 ng in 200 nL), and its effects were recorded on BP and water intake. Baseline mean arterial pressure (MAP) and heart rate, 7 days after infection, were not significantly different between Ad-eGFP (MAP: 111±3 mm Hg; heart rate: 605±9 bpm) and Ad-hACE2-eGFP (MAP: 113±3 mm Hg; heart rate: 617±26 bpm; P>0.05) groups. Figure 3A illustrates the typical pressor response induced by ICV Ang II in a conscious mouse previously infected with Ad-eGFP showing a rapid increase in BP developing in the minutes following ICV injection and slowly returning to baseline. Ad-eGFP pretreatment did not affect the peak effect and duration of the ICV Ang II pressor response.¹⁴ However, mice infected with Ad-hACE2-eGFP exhibit a significant reduction in Ang II–mediated pressor response with decreases in both amplitude (ΔMAP: Ad-hACE2-eGFP 8±2 versus Ad-eGFP 18±2 mm Hg; P<0.01) and duration of the effect (ΔMAP: Ad-hACE2-eGFP 9±2 versus Ad-eGFP 16±2 minutes; P<0.05) (Figure 3B and 3C). This reduction in the Ang II pressor response was not prevented by pretreatment with D-Ala⁷-Ang-(1-7) (ΔMAP: Ad-hACE2-eGFP 5±3 versus Ad-eGFP 14±2 mm Hg), suggesting that hACE2 expression in the SFO inhibits Ang II–induced pressor response by decreasing Ang II levels rather than involving an Ang-(1-7)–mediated mechanism. In addition, Ad-hACE2-eGFP pretreatment had no effect on the pressor response mediated by the muscarinic agonist carbachol (100 ng ICV in 200 nL) (ΔMAP: Ad-hACE2-eGFP 14±3 versus Ad-eGFP 18±7 mm Hg; P>0.05), confirming the selectivity of hACE2 for angiotensin peptides. Finally, the decrease in Ang II–mediated pressor response observed in the Ad-hACE2-eGFP group was correlated to an increase in enzyme activity in these mice (Figure 3D).

To confirm the ability of SFO-targeted hACE2 expression in preventing local Ang II–mediated effects, we assessed the Ang II–mediated drinking response following adenovirus infection.
pretreatment. Ad-hACE2-eGFP significantly blunted the water intake behavior resulting from central administration of Ang II (Ad-hACE2-eGFP: 5 ± 3 versus Ad-eGFP: 41 ± 6 seconds; P < 0.001) (Figure 4A).

To assess whether disruption of local Ang II levels by hACE2 in the SFO is able to modify distant physiological mechanisms, we analyzed baroreflex sensitivity in mice following virus infection. No significant changes were observed in SBRS 7 days after infection (up sequences: Ad-hACE2-eGFP 2.0 ± 0.5 versus Ad-eGFP 2.2 ± 0.4; down sequences: Ad-hACE2-eGFP 1.5 ± 0.2 versus Ad-eGFP 1.6 ± 0.2 ms/mm Hg; P > 0.05; Figure 4B), suggesting that disruption of Ang II signaling in the SFO is unable to alter baroreflex mechanisms originating in the brainstem.

ACE2 and AT1 Receptor Expression

To gain insight of the mechanism by which ACE2 prevents Ang II–mediated pressor and drinking responses, we investigated the effects of ACE2 overexpression on AT1 receptors regulation. This choice was motivated by the inability of D-Ala7-Ang-(1-7) to block Ad-hACE2-eGFP–mediated effects and by previous studies showing that Ang-(1-7) has hypotensive effects in hypertensive but not in normotensive animals.

In vitro, overexpression of ACE2 in neuro-2A cells resulted in a 70% reduction (0.30 ± 0.03) of AT1 protein expression compared with Ad-eGFP (1.00 ± 0.08; P < 0.001) (Figure 5A). Similarly, hACE2 overexpression in neuro-2A cells significantly reduced the Ang II receptor binding (B) and immunostaining (C and D) compared with Ad-eGFP treated cells. *P < 0.05, ***P < 0.001 vs Ad-eGFP.

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extracts from Ad-hACE2-eGFP–treated mice (Figure 6B). Finally, AT1 receptor immunostaining (Figure 6C) was significantly reduced in the core of the SFO following ACE2 overexpression. Altogether, our data suggest that ACE2 exerts its modulatory effects partly through downregulation of the AT1 receptor expression, thus preventing Ang II–mediated pressor and drinking responses.

Discussion

Overactivity of the RAS has been implicated in the development and maintenance of several cardiovascular diseases, and participation of the brain RAS to such diseases is now well recognized.1,2 However, studying the brain RAS has been limited by the difficulty of unmasking brain-specific mechanisms and avoiding systemic RAS-mediated effects.2 Nevertheless, important data have emerged, such as the role of key forebrain and brainstem regions in which activation of angiotensin pathways leads to an increase in sympathetic tone. The difficulty in understanding how these pathways may interact has recently been increased by the discovery of new players in the RAS, both in the periphery and central nervous system.7,9 Consequently, new tools are needed to face the challenge of an evolving brain RAS. In this study, we developed a new viral vector expressing hACE2 and unmasked a role for ACE2 in buffering the Ang II–mediated pressor and drinking responses originating in the SFO, suggesting a role for this enzyme in the central regulation of cardiovascular function and volume homeostasis.

To overcome the limitations associated with studying the brain RAS, scientists have used brain-targeted overexpression of RAS genes by generation of transgenic animals,14,23,24 gene delivery,18,25,26 or a combination of both.15,27 These approaches have been successful in unmasking physiological responses otherwise below detection levels. Classic methods of gene therapy for the brain include the use of adenovirus,28 lentivirus,22,26 or adenoassociated virus.29 Unlike lentivirus, targeting essentially neurons when administered in the brain, adenovirus vectors have no tissue tropism, therefore preventing random insertional mutagenesis. Although we did not assess inflammation resulting from virus injection, previous work using similar vectors and titers in the brain,15,27 added to the lack of abnormal behavior in our infected mice, ruled out this possibility. Finally, the rapid (as early as 3 days) and efficient expression of hACE2 makes Ad-hACE2-eGFP an attractive tool to manipulate ACE2 expression and activity in vitro and in vivo.

The main goal of this study was to assess the effects of ACE2 overexpression on Ang II–mediated physiological responses. Our data provide the first evidence that ACE2 overexpression in the forebrain counters Ang II–mediated responses partly by downregulating AT1 receptors expression. ICV administration of Ang II is responsible for powerful pressor and bradycardic responses preceding water intake, all resulting from the stimulation of AT1 receptors located in the SFO and other nuclei of the lamina terminalis.1,22 Our data show that ACE2 overexpression to the forebrain, essentially the SFO, inhibited both pressor and drinking responses resulting from ICV administration of Ang II. This could result from: (1) a decrease in Ang II levels attributable to ACE2-mediated conversion of Ang II into Ang-(1-7), thus leading to a lesser stimulation of AT1 receptors; or (2) a decrease in Ang II levels associated with increased Ang-(1-7) levels, leading to the activation of an Ang-(1-7) receptor. Although Ferreira et al observed a slight decrease in water intake in transgenic rats with high plasma Ang-(1-7) levels,33 although Ang-(1-7) was shown to alter the bradycardic component of the baroreflex,21 the participation of Ang-(1-7) in the reduction of Ang II–mediated BP and water intake in our experiments seems unlikely. Indeed, the vasoactive properties of Ang-(1-7) in vivo have been primarily reported in pathophysiologic conditions, such as hypertension or myocardial infarction, and this peptide has minimal effects in normotensive animals.9 Accordingly, failure to restore Ang II–mediated pressor and drinking responses following pretreatment with an Ang-(1-7) receptor blocker may be expected. Moreover, the fact that we observed no change in either arm of the baroreflex curve suggests that decreased Ang II–mediated pressor and drinking responses only results from lessened stimulation of the Ang II signaling pathways. These observations are consistent with the ability of ACE2 to hydrolyze Ang II with a high catalytic efficiency (kcat/Km = 1.9×105 mol/L·sec−1).8 Finally, our conclusions are supported by a recent study in which a lentivirus coding for the murine ACE2 was delivered to the rostral ventrolateral medulla of spontaneously hypertensive rats, resulting in a transient decrease in BP and heart rate 4 weeks postinfection.26 Because injection of Ang-(1-7) in the rostral ventro-
lateral medulla has previously been shown to increase BP.\textsuperscript{34} These data suggest that the BP decrease was not mediated by Ang-(1-7) and is likely to result from a reduction in Ang II signaling pathway.

Although the unaltered baroreflex sensitivity following hACE2 overexpression in the SFO probably suggests that modulation of the RAS is restricted to the region infected by the virus, our data show that hACE2 is also a target for sheddases, thus releasing the enzyme in the cell environment. In the case of the brain, because the secreted form conserves its activity, we cannot rule out the possibility that secreted ACE2 could travel from the interstitial to the cerebrospinal fluid, allowing the enzyme to modulate Ang II levels and central signaling pathways throughout the brain.

The SFO exerts a key role in the central regulation of BP and volume homeostasis.\textsuperscript{15,27,32} Indeed, the lack of a blood–brain barrier renders it sensitive to circulating peptides, such as angiotensins, that can reach the brain and stimulate local receptors to exert central effects in addition to their peripheral effects. Not only is the SFO sensitive to systemic peptides, but it is also a pivotal site for synthesis of Ang II involved in generating cardiovascular and drinking responses.\textsuperscript{15,27} In addition, the SFO projects and receive projections from several brain regions, including paraventricular and supraoptic nuclei, nucleus of tractus solitarius, and ventrolateral medulla.\textsuperscript{32} Overexpression of ACE2 in the SFO could potentially result in the alteration of several downstream and upstream neuronal networks. For example, as a result to ACE2 overexpression in the SFO, neuronal activation could be reduced in the PVN, thus participating in the reduction of both pressor and drinking responses. However, lack of hACE2 expression in the PVN rules out a direct involvement.

Interestingly, we observed that prevention of Ang II–mediated pressor and drinking responses, by ACE2 overexpression, was associated with a downregulation of AT\textsubscript{1} receptor expression in the SFO. These data suggest that not only does ACE2 overexpression reduce the amount of Ang II available to stimulate its receptors, but it also further impairs Ang II downstream signaling by limiting the number of AT\textsubscript{1} receptors mediating these signaling pathways. Whether this decrease in AT\textsubscript{1} receptor expression on the cell membrane resulted from internalization or gene regulation remains to be determined. This could be mediated by Ang-(1-7), as suggested previously,\textsuperscript{35} although our experiments seem incompatible with formation of the micromoles of Ang-(1-7) necessary to produce this response. Alternatively, it is conceivable that longer stimulation of Ang-(1-7) release could lead to such downregulation.\textsuperscript{36} More recent studies have reported an inhibitory effect of AT\textsubscript{1} receptors on ACE2 gene and protein expression\textsuperscript{37,38}; however, assessing the role of ACE2 on other RAS components had been limited to date by the lack of tools available to modulate ACE2 expression. Ad-hACE2-eGFP was determinant in showing for the first time that ACE2 is able to regulate other components of the RAS.

In summary, these results demonstrate that overexpression of ACE2 in the SFO plays a pivotal role in the regulation of BP and volume homeostasis by modulating both amounts of Ang II and AT\textsubscript{1} receptors available to stimulate downstream signaling pathways. Moreover, we speculate that ACE2-mediated formation of Ang-(1-7) may only be relevant in pathophysiological conditions. Finally, this study provides proof in principle of a therapeutic role for ACE2 gene therapy and, more generally, validates ACE2 as a new target for the treatment of conditions involving a dysfunctional RAS, such as hypertension and other cardiovascular diseases.

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Disclosures

None.

References


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Material and Methods

Adenovirus Generation

The Ad-hACE2-eGFP virus was developed in collaboration with the University of Iowa Gene Transfer Vector Core. Briefly, the ACE2 pcDNA3.1 vector (kind gift of Dr Curt D. Sigmund, University of Iowa) was digested with XbaI and Pmel to excise the 2418 bp human ACE2 (hACE2) fragment (accession number: AF291820). This fragment was then cloned into a pacAd5 CMV IRES eGFP pA shuttle (University of Iowa Gene Transfer Vector Core) (online data supplement 1A). The resulting construct was then used to generate the hACE2-eGFP adenovirus as described previously.1

Cell culture and adenovirus infection

Neuro-2A mouse neuroblastoma cells (ATCC Manassas, VA) were grown in minimum essential medium (MEM)(GIBCO®, Invitrogen, Carlsbad, CA) with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% fetal bovine serum (FBS)(GIBCO®) at 37°C under a humidified 95% and 5% (v/v) mixture of air and CO₂. Cells were grown into 6-well plates at a density of 2x10⁶ cells/well. After 24 hr, cells were incubated in a low FBS medium (2%) in the presence of Ad-hACE2-eGFP (10, 50, 100 MOI) virus or Ad-eGFP control virus (100 MOI) for 6 hr then returned to a 10% FBS medium. On the
fourth day post infection cells were observed using a fluorescence microscope (Olympus, IX81). Then media and cells were collected and assayed for ACE2 western blot and activity, AT_1 immunohistochemistry and receptors binding experiments.

**In-vivo adenovirus intracerebroventricular (icv) injection**

All procedures were approved by the Institutional Animal Care and Use Committee at Louisiana State University Health Sciences Center-New Orleans. Male C57Bl/6J mice, 8-10 weeks old, were purchased from Charles River Laboratories (Wilmington, MA) and housed in cages (12-hour light/dark cycle) with normal chow and water. Following anesthesia (Sodium pentobarbital 50 mg/Kg i.p.), mice were placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL) and Ad-hACE2-eGFP or Ad-eGFP were injected icv (2x10^6 pfu, 200 nL) using a pressure injector (PicospritzerII). Upon recovery, mice were returned to their home cage. Mice were anesthetized and intracardially perfused with saline and 4% paraformadehyde at 3, 7, 14 and 28 days post infection. The brain was then removed and stored overnight in 20% sucrose before being sectioned coronally (30 µm) in a cryostat. Sections were mounted on slides and GFP fluorescence was visualized using a fluorescence microscope (Olympus, IX81). In additional experiments, brains were frozen at -80˚C before the SFO be dissected and used for ACE2 and, AT_1 western blot, AT_1 receptor binding experiments and ACE2 activity assay as described below.
**AT₁ receptor binding**

The AT₁ receptor density was determined in enriched plasma membrane preparations from subfornical organs and neuro-2A cells as described previously,²,³ following infection with Ad-hACE2-eGFP or Ad-eGFP. Briefly, SFOs (n=20 per group) were dissected, homogenized with a Polytron in buffer containing 150 mM NaCl, 5 mM EDTA, 20 mM Tris-HCl (pH=7.4 with HCl) and supplemented with Complete Mini protease inhibitor mixture (Roche Applied Science, Mannheim, Germany). Neuro-2A cells were processed in the same manner, but with lysis buffer containing 5 mM Tris-HCl, 5 mM EGTA and 5 mM EDTA (pH 7.4) supplemented with Complete Mini protease inhibitor mixture. The enriched plasma membrane was obtained by centrifugation at 100,000 x g for 60 min and the pellet was re-suspended in 500 µl of binding buffer containing 50 mM Tris-HCl, pH 7.4, 0.6 mM EDTA, and 5 mM MgCl₂. The membrane suspension (100 µg/reaction) was incubated with 100 pM ¹²⁵I-[Sar¹,Ile⁸]Ang II (Perkin Elmer, specific activity: 2200 Ci/mmol) for 2 hr at room temperature and the bound radioactivity was separated by vacuum filtration using glass filters (Whatman®, Schleicher & Schuell). The non-specific binding was determined in the presence of 5 µM of non-radioactive Ang-II and the radioactivity was measured in a γ-counter.

**Western blot**

Three days after infection, cells and culture media were collected separately and centrifuged at 4000 rpm, 4°C for 5 min. Supernatants were transferred to clean
tubes while cell pellets were incubated in 100 µL 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). SFO tissues were homogenized with a glass pestle in the same lysis buffer. 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). Cell culture media (10 µL), cell lysates (10 µg) and SFO plasma membrane protein extracts (5 µg) were mixed with SDS-PAGE sample buffer (0.125 M 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 mM NaH₂PO₄, 8.09 mM Na₂HPO₄, 145 mM 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 or rabbit-anti-mouse AT₁ antibody (SC-1173, Santa Cruz, CA, 1:200) 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) or goat-anti-rabbit IgG-HRP (NEF812, Perkin Elmer, 1:5000) and goat anti-mouse beta-actin antibody (ab8229, abCAM, 1:5000) for 45 min at room temperature. Specific bands were detected by chemiluminescence according to the manufacturer's instructions (ECL®, Perkin Elmer, Boston, MA)
and quantitated by laser densitometry (FujiFilm, ImageReader version 1.2). Bands corresponding to hACE2 were normalized to β-actin for cell and SFO tissue lysates; expressed as pixel intensity for the cell culture media and membrane protein extract.

ACE2 activity

Three days after infection, cells and culture media were collected separately and centrifuged at 4000 rpm, 4°C for 5 min. Supernatants were transferred to clean tubes while cell pellets were incubated in 100 µL 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. Hypothalamic regions were collected at 3, 7, 14 and 28 days and SFOs were dissected at 7 days post infection with the Ad-hACE2-GFP or Ad-GFP viruses (2x10⁶ pfu icv), homogenized in 1 mL reaction buffer and processed as described above.

ACE2 activity measurement was carried out in the presence of captopril to eliminate any contribution by endogenous 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. ACE2 activity was measured in both supernatants and cell culture media. Cell and hypothalamus lysates containing
10 and 100 µg of protein, respectively, 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 is normalized for total protein or volume of media.

**Immunohistochemistry**

Immuno-fluorescent detection of human ACE2 and AT₁ receptor protein was performed in neuro-2A cells 48 hr post adenovirus infection or in the SFO, 7 days after infection with Ad-hACE2-eGFP (n=5) or Ad-eGFP (n=5). Mice were euthanized with an overdose of Nembutal and were perfused transcardially with PBS (0.1M, pH 7.4) during 2 min followed by 4% paraformaldehyde in PBS (0.1M, pH 7.4) for 10 min. The brain was then removed, post fixed for 2 hr in 4% paraformaldehyde in PBS (0.1M, pH 7.4) and then placed in 30% sucrose solution overnight. Next, 16 µm coronal sections were collected on slides and permeabilized with 0.2% Tween-20® (15 min), while non specific binding was
blocked with 5% bovine serum albumin (BSA, 1 hr). Sequential sections were subjected to immunohistochemistry using a goat anti-human ACE2 antibody (SC-17720, Santa Cruz) or a rabbit anti-mouse AT1 receptor antibody (SC-1173, Santa Cruz, CA) 1:50 dilution for 48 hr. Sections were incubated at room temperature with the secondary antibody: a rhodamine-conjugated donkey-anti-goat IgG (ab7122, abCAM, 1:100 dilution, 1 hr) for hACE2 and a rhodamine-conjugated donkey anti-rabbit IgG for AT1 (ab7080, abCAM, 1:100 dilution, 1 hr). At the end of the protocol, sections were incubated for 10 min in DAPI (4’,6-diamidino-2-phenylindole, dihydrochloride) staining for double-stranded DNA visualization. Immunostaining was detected using fluorescence (Olympus, IX81). Finally, primary antibody was omitted in some incubation to check for auto-fluorescence.

For AT1 immunohistochemistry in the SFO, mouse brains were fixed as above and 30 µm coronal sections were collected on slides and non-specific binding was blocked with 5% bovine serum albumin (BSA) for 1 hr. Sequential sections were subjected to immunohistochemistry using an AT1 receptor antibody (SC-1173, Santa Cruz, CA) 1:50 dilution for 36 hr. Sections were incubated at room temperature with the secondary antibody a donkey-anti-rabbit IgG-HRP (NEF812, Perkin Elmer) 1:100 dilution for 1 hr. At the end of the protocol, color was developed by incubation with DAB substrate, according to the manufacturer (Vector Laboratories, SK-4100). Images were captured using a brightfield microscope (Nikon Eclipse E600). Additionally, either or both primary and
secondary antibodies were omitted in the incubation to check for unspecific staining.

**Physiological recordings**

C57Bl/6J mice (n=25) were anesthetized as described above and surgically instrumented with an icv canula (relative to bregma: 0.3 mm posterior, 1 mm lateral and 3.0 mm ventral) and a radiotelemetry probe (PA-C10, DSI, St Paul, MN) for chronic blood pressure (BP) recording. Mice were then injected icv with Ad-hACE2-eGFP (n=13) or Ad-eGFP (n=12) (2x10^6 pfu, 200 nL) and returned to their home cage. On the 7th day post infection, BP was continuously recorded for 30 min in conscious freely moving mice preceding random icv injection with Ang-II (200 ng, 200 nL), carbachol (100 ng, 200 nL) or the Ang-(1-7) receptor blocker, D-Ala^7^-Ang-(1-7) (200 fmol, 200 nL) prior to icv Ang-II and then monitored for an additional 30 min. Water intake was monitored in each case by recording the time spent drinking during the 15 min following Ang-II administration. From the baseline recording, spontaneous baroreceptor reflex sensitivity (SBRS) was calculated using the sequence method as described previously.5

**Statistical Analysis**

Data are expressed as mean ±SEM. Data were analyzed by Student t test or ANOVA (after Bartlett test of homogeneity of variance), followed by Newman–Keuls correction for multiple comparisons between means. Statistical
comparisons were performed using Prism4 (GraphPad Software, San Diego, CA). Differences were considered statistically significant at $P<0.05$.


Online Figure Legends:

Online Figure I. Ad-hACE2-eGFP expression in neuro-2A cells. Human ACE2 cDNA (A1) was inserted into pacAd5 CMV IRES eGFP pA shuttle vector (A2) upstream of an eGFP reporter gene. This final construct (A3), allowing hACE2 and eGFP expression as non-fusion proteins under the control of the same CMV promoter, was then used to generate the hACE2-eGFP adenovirus. Neuro-2A cells (mouse neuroblastoma, ATCC) were infected with Ad-hACE2-eGFP (10-100 MOI) or the control Ad-eGFP virus (100 MOI) at a density of 2x10^5 cells/well. Brightfield and GFP fluorescence pictures (B) were taken 72 hr post infection (20X). GFP expression was dose-dependently increased following Ad-hACE2-eGFP virus infection. Cell culture media (C) were blotted with anti hACE2 ectodomain or N-terminal antibodies. The hACE2 protein appears properly processed and released into the cell culture medium. “P” indicates a positive control (whole cell lysate 72 hours after 100 MOI Ad-hACE2-eGFP infection) on the anti-hACE2 N-terminal antibody blot.

Online Figure II. Time-dependent Ad-hACE2-eGFP expression in the SFO. SFO sections were collected and processed for eGFP fluorescence 3, 7, 14 and 28 days after virus infection. ICV-delivered Ad-hACE2-eGFP induced a significant increase of eGFP fluorescence at 3 and 7 days. Despite the persistence of few cells still harboring eGFP in the wall of the lateral ventricle (data not shown), fluorescence had disappeared at 14 and 28 days after Ad-hACE2-eGFP infection. ACE2 activity (graph) in the hypothalamus following Ad-
eGFP (GFP) or Ad-hACE2-eGFP (ACE2) infection, was positively correlated to eGFP fluorescence in the SFO. Statistical significance: ***$P<0.001$ vs. Ad-eGFP.

**Online Figure III. eGFP and hACE2 expression in both neurons and glial cells.** Seven days after virus infection, SFO sections were collected and processed for hACE2 immunohistochemistry. High magnification (100X) pictures were captured for both eGFP (green) and hACE2 immunofluorescence (red) and show that eGFP and hACE2 are co-localized to the same cells. Human ACE2 (B and D) was detected in both neurons (B) and glial cells (D), although hACE2 expression was weaker in glia (D).
Online Figure I

A

B

no virus

Ad-eGFP
100 MOI

Ad-hACE2-eGFP
10 MOI

Ad-hACE2-eGFP
50 MOI

Ad-hACE2-eGFP
100 MOI

C

N-17 terminal

ectodomain

Ad-eGFP

Ad-hACE2-eGFP

0 100 0 0 0 0

0 0 10 50 100
Online Figure II

ACE2 activity (AFU/mg.min)
Online Figure III