Overexpression of Angiotensin-Converting Enzyme 2 in the Rostral Ventrolateral Medulla Causes Long-Term Decrease in Blood Pressure in the Spontaneously Hypertensive Rats

Masanobu Yamazato, Yoriko Yamazato, Chengwen Sun, Carlos Diez-Freire, Mohan K. Raizada

Abstract—The rostral ventrolateral medulla (RVLM) is a relay point that provides supraspinal excitatory input to sympathetic preganglionic neurons in the regulation of blood pressure. The importance of the RVLM is further highlighted by observations that an increase of RVLM sensitivity to angiotensin II and enhanced sympathetic activity are associated with hypertension. Angiotensin-converting enzyme 2 (ACE2) has been shown to be central in maintaining the balance between vasoconstrictor activity of angiotensin II with vasoprotective action of angiotensin-(1-7) in the peripheral system. However, its role in central control of blood pressure in the RVLM is yet to be investigated. Thus, our objective in this study was to compare ACE2 expression in the RVLM of Wistar–Kyoto rats and spontaneously hypertensive rats and to determine whether RVLM ACE2 is involved in blood pressure control. ACE2 immunoreactivity was diffusely distributed in many cardiovascular regulatory neurons, including the RVLM. Western blot analysis revealed a 40% decrease in ACE2 in the RVLM of spontaneously hypertensive rat compared with Wistar–Kyoto rats. Lentiviral-mediated overexpression of ACE2 (lenti-ACE2) was used to determine whether a decrease in ACE2 in the RVLM is associated with hypertensive state. Bilateral injection of lenti-ACE2 resulted in a long-term expression of transgenic ACE2. This was associated with a decrease in mean arterial pressure exclusively in the spontaneously hypertensive rat (141±4 mm Hg in lenti-GFP versus 124±5 mm Hg in lenti-ACE2) and heart rate (304±7 bpm in lenti-GFP versus 285±5 bpm in lenti-ACE2). These observations demonstrate that overexpression of ACE2 overcomes its intrinsic decrease in the RVLM and decreases high blood pressure in the spontaneously hypertensive rat.

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Key Words: blood pressure ■ Wistar–Kyoto rat ■ RVLM ■ gene transfer ■ lentiviral vector

Increasing evidence indicates that a hyperactive brain rennin–angiotensin system (RAS) is critical in the development and maintenance of hypertension. The rostral ventrolateral medulla (RVLM) is 1 of the brain areas that coordinate the propagation of angiotensin (Ang) II signals leading to hyperactivity of this hormone in hypertension.1 This conclusion is supported by observations that the RVLM is considered the final relay point before transmission of Ang II signals to periphery2 and that Ang II type 1 receptors and Ang II sensitivity in the RVLM of spontaneously hypertensive rats (SHRs) and other rat models of hypertension are increased.3–5 Thus, regulation of the RAS activity in the RVLM is critical in a long-term regulation of neural control of blood pressure and other cardiovascular functions.

The recent discovery of angiotensin-converting enzyme 2 (ACE2) provides a novel target for a chronic regulation of the brain Ang II system with implications on a long-term beneficial outcome to the cardiovascular system.5,7 ACE2 has been implicated in maintaining the balance between vasoconstrictor activity of Ang II with vasoprotective actions of Ang-(1-7) and other related peptides in the peripheral system.8–10 As a result, it has been suggested that a decrease in intrinsic ACE2 would lead to hypertension and related pathophysologies, whereas its overexpression or activation would be beneficial in these conditions. This conclusion is supported by evidence that a decrease in ACE2 is associated with cardiac and renal pathophysiologies induced in cardiovascular disease and hypertension.11 Our observations have demonstrated that ACE2 overexpression attenuates development of hypertension-induced cardiac remodeling in the Ang II infusion rat model of hypertension.12 In addition, ACE2 gene transfer both lowers blood pressure (BP) and improves cardiac pathology in the SHR.13 In spite of these dramatic beneficial effects of ACE2 in the peripheral system and the observation that ACE2 is present in various cardiovascular regulatory brain areas,14,15 the role of this enzyme in neural control of BP has not been delineated. These observations, coupled with the importance of the RVLM in central cardiovascular regulation, led us to propose the following hypothesis: ACE2 expression is decreased in the RVLM in hyper-
tensive rats, and, thus, its overexpression would lead to beneficial effects on BP. The present study was conducted to support or refute this hypothesis. Our observations demonstrate that overexpression of ACE2 in the RVLM overcomes its intrinsic decrease and partially lowers high BP in the SHR.18

Methods

Animals

Twelve-week-old male SHRs and Wistar-Kyoto (WKY) rats were purchased from Charles River Laboratories (Wilmington, Mass). Rats were housed individually and kept on a 12:12 h light–dark cycle in a climate-controlled room. Rat chow (Harlan Tekland) and water were provided ad libitum. All of the animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Florida.

Abdominal Aorta Cannulation

Rats were anesthetized with inhaled isofluorane (3%) in all of the surgical procedures. Radiotelemetric pressure transducers (Data Sciences International) consisting of a fluid-filled catheter attached to a PA-C40 transmitter were implanted into the abdominal aorta as described previously.16 Before implantation, the aorta was clamped proximally and the catheter inserted and secured with medical adhesive. During experiments, BP and heart rate (HR) were recorded every 5 minutes for an average of 10 s from 10:00 AM to 11:00 AM, 3 times a week. Weekly data from each rat were averaged for analysis. Rats were received an IM injection of 50 mg/kg body weight of ampicillin for prophylaxis and were allowed to recover for 1 week after all of the surgeries.

Implantation of Bilateral Guide Cannula to the RVLM

Skin overlying the midline of the skull was incised, and a small hole was drilled bilaterally on the dorsal surface of the cranium according to the following coordinates: 1.9 mm lateral to the midline and 3.0 mm posterior to the lambdoid suture. A stereotaxic frame was used to position a 22-gauge double guide cannula (PlasticsOne), and the guide cannula was lowered 6 mm below the skull to place the tip 4 mm above the RVLM. The cannula was secured to the skull with dental resin assisted by 3 screws secured in the skull.16

Production of Lentiviral-Mediated Overexpression of ACE2 Viral Particles

Lentiviral particles containing enhanced green fluorescent protein (GFP; EF1α-IRES-EGFP, lenti-GFP) or murine ACE2 (EF1α-ACE2-IRES-EGFP, lenti-ACE2) were prepared by methods described previously.17,18 Viral medium containing lenti-GFP or lenti-ACE2 was collected, concentrated, and titred. Concentration of viral particles was determined with the use of HIV-1 p24 antigen ELISA assay (Beckman Coulter) following the manufacturer’s instructions. Efficacy of lenti-ACE2 in producing active ACE2 enzyme has been established previously.12,13

In Vivo Gene Delivery Into the RVLM

Before injection of lentiviral vectors, rats received L-glutamate (2 nmol/200 nL) injections through the bilateral guide cannula to establish that the injection site was the pressor area of the ventrolateral medulla.19 Rats that expressed a rapid pressor response of 20 mm Hg to L-glutamate were used for the study. Animals were divided into 2 groups: control rats received lenti-GFP, and experimental rats received lenti-ACE2 into the bilateral RVLM. Animals receiving lenti-ACE2 into the bilateral trigeminal nucleus served as non-RVLM controls. Lentiviral particles (3 × 107 transfection units) in 250 nL of cerebrospinal fluid were injected into each injection site at 10-minute periods using a 32-gauge Hamilton syringe (0.5 μL).

Histological Examination

Fixation and immunostaining procedures used in this study have been described previously.20 In brief, rats were perfused transcardially with 50 mL of 0.9% NaCl followed by 50 mL of 4% paraformaldehyde in PBS. The brain stem was removed, postfixed in 4% paraformaldehyde solution for 1 hour, and transferred to a phosphate buffer containing 20% sucrose (pH 7.4). Frozen brain tissues were sectioned in the coronal plane (20 μm). Sections without immunostaining were used to examine GFP expression. Microinjection sites were identified by the GFP expression using a Zeiss Axioplan 2 florescence microscope and correlated to anatomic structures of the rat medulla oblongata according to the atlas.21 For immunostaining, sections were first incubated with 0.3% H2O2 in methanol for 15 minutes, followed by incubation with 1.5% goat serum in PBS containing 0.3% Triton X100 for 60 minutes. They were incubated with rabbit polyclonal antibody to ACE2 (1:50, sc-20998, Santa Cruz Biotechnology) or another rabbit polyclonal antibody to ACE2 (1:500, GTX15348, GeneTex) containing 0.3% BSA in PBS containing 0.3% Triton X100 overnight at 4°C followed by incubation with biotinylated goat anti-rabbit IgG for 120 minutes and avidin–biotin–peroxidase complex reagents for 60 minutes and stained with diaminobenzidine solution for 8 minutes according to the manufacturer’s instructions (Vector Laboratories). Each step was followed by washing the sections with PBS containing 0.3% Triton X100. Sections incubated without primary antibody were used as negative controls. To further examine the specificity of the antibodies, immunohistochemical staining was also performed after antibody preabsorption with excess ACE2 peptide (GTX15352, GeneTex, Inc) with modified manufacturer’s instructions. The primary antibody, GTX15348 (1:500), was preabsorbed with 2 μg/mL of the ACE2 peptide including 1% BSA for 1 hour at room temperature and further incubated overnight at 4°C. After centrifuging the solution, the supernatant was used in the same immunolabeling procedure.

Western Blot Analysis

Using a Harris Micro-Punch (1 mm, Electron Microscopy Sciences), both sides of the RVLM were punched out from the 1–mm–thick section as described by Comer et al.22 Thirty-two RVLM punches were obtained from medulla oblongata of WKY rats (n = 8) and SHRs (n = 8), and the 4 punches from each strain were pooled and subjected to protein isolation protocol (Santa Cruz Biotechnology). Twenty micrograms of protein were run on a 12% SDS-PAGE, and the proteins were transferred onto a nitrocellulose membrane. After 1 hour blocking with 5% milk in Tris-buffered saline-Tween, the membrane was probed with the rabbit polyclonal antibody to ACE2 (1:200 in 1% milk/Tris-buffered saline-Tween) overnight. Membrane was washed 3 times for 10 minutes in Tris-buffered saline-Tween and incubated with anti-rabbit IgG–horseradish peroxidase–conjugated secondary antibody (1:5000) for 1 hour. After final washes, the membrane was incubated with chemiluminescent agent for 1 minute and the exposed to film to visualize protein bands. α-Tubulin bands were analyzed in parallel as a loading control.

Statistical Analysis

Values are expressed as mean±SEM. Comparisons between experimental groups were analyzed using ANOVA and Student’s t test. A value of P<0.05 was considered significant.

Results

Our first objective was to determine whether ACE2 is localized in cardiovascular-relevant brain areas of rats, and immunostaining was performed to accomplish this goal. Figure 1A shows that ACE2 immunoreactivity was diffusely distributed in all of the cardiovascular-relevant areas in the medulla oblongata: the area postrema (Figure 1A, panels b and f), the dorsal motor nucleus of the vagus (Figure 1A, panel b), the nucleus tractus solitarius (Figure 1A, panels c and g), the nucleus ambiguus (Figure 1A, panel d), and the...
ventrolateral medulla (Figure 1A, panels d and h). Nuclei of cranial nerves in the medulla oblongata were also immunostained for ACE2. The immunoreactivity in the RVLM neurons was observed in cytoplasm of neurons and its proximal dendrite (Figure 1A, panel h). The immunoreactivity in the RVLM neurons was observed in cytoplasm of neurons (arrowhead) and its proximal dendrite (arrow). The scale bar denotes 100 μm in the top and 20 μm in the bottom. B, Coronal sections of the medulla oblongata were immunostained with polyclonal anti-ACE2 antibody (GTX15348, GeneTex) as described in the Methods section. The low-magnification photographs of sections show negative immunostaining controls consisted of exclusion and preadsorption of the primary antibodies for ACE2 (a and b, respectively). The corresponding low-magnification photograph (c) shows the distribution of ACE2 immunoreactivity in the ventrolateral medulla. The distribution of ACE2 immunoreactivity using this antibody was similar to that of immunoreactivity using the Santa Cruz antibody. The high-magnification photograph shows ACE2 immunoreactivity in the RVLM neurons (d). The scale bar denotes 100 μm in a, b, and c, and 20 μm in d.

Figure 1. ACE2 immunoreactivity in the rat medulla oblongata. A, Coronal sections of the medulla oblongata were immunostained with polyclonal anti-ACE2 antibody (sc-20998, Santa Cruz Biotechnology) as described in the Methods section. Sections incubated without primary antibody were used as negative control (a and e). The low-magnification photographs of sections (top) show the distribution of ACE2 immunoreactivity in the caudal part of medulla oblongata including area postrema (AP) and the dorsal motor nucleus of the vagus (b), the NTS (c), and the ventrolateral medulla, including nucleus ambiguous and RVLM (d). The high-magnification photographs of sections (bottom) show ACE2 immunoreactivity of neurons in the AP (f), neurons (arrowhead) and neuropils (arrow) of the NTS (g) and neurons of the RVLM (h). The immunoreactivity in the RVLM neurons was observed in cytoplasm of neurons (arrowhead) and its proximal dendrite (arrow). The scale bar denotes 100 μm in the top and 20 μm in the bottom. B, Coronal sections of the medulla oblongata were immunostained with polyclonal anti-ACE2 antibody (GTX15348, GeneTex) as described in the Methods section. The low-magnification photographs of sections show negative immunostaining controls consisted of exclusion and preadsorption of the primary antibodies for ACE2 (a and b, respectively). The corresponding low-magnification photograph (c) shows the distribution of ACE2 immunoreactivity in the ventrolateral medulla. The distribution of ACE2 immunoreactivity using this antibody was similar to that of immunoreactivity using the Santa Cruz antibody. The high-magnification photograph shows ACE2 immunoreactivity in the RVLM neurons (d). The scale bar denotes 100 μm in a, b, and c, and 20 μm in d.

To determine whether a decrease in ACE2 in the SHR RVLM is associated with hypertensive state, lentiviral-mediated gene transfer of ACE2 was used. BP and HR were recorded 3 times a week using radiotelemetry in a conscious state for ≤5 weeks after the gene transfer. First, we evaluated the sites of transgene expression and the transgene efficiency. Location of gene transfer was examined by green fluorescence, whereas transgene expression was determined by ACE2 immunohistochemistry. Figure 3A shows a brain stem section of the SHR that received lenti-ACE2. GFP expression was remarkably restricted to the area of RVLM. Figure 3B shows a comparable section with ACE2 immunohistochemistry. It demonstrates that ACE2 expression was also restricted and was increased in the neurons of RVLM when compared with its intrinsic levels. Figure 3C presents a section of RVLM that was first examined for GFP fluorescence followed by immunostaining for ACE2. It shows that most of the cells that express GFP also show strong staining for ACE2. This observation, coupled with the fact that both GFP and ACE2 genes are driven by the same promoter, indicates that GFP and ACE2 are present in the same cells. Figure 4 shows ACE2 protein levels of lenti-ACE2 injected site. Six weeks after the lenti-ACE2 injection, ACE2 protein levels in the RVLM were increased by 45% in the lenti-ACE2–injected SHR compared with the noninjected SHR; however, the protein levels were still 22% lower than that of WKY rats. Figure 5 shows the effect of ACE2 overexpression on mean arterial pressure (MAP) and HR. Lenti-ACE2–injected SHR showed a time-dependent decrease in MAP as early as 2 weeks after gene transfer, which reached statistical significance by week 4. This decrease persisted for an additional week at the time of termination of the experiment (Figure 5). The decrease in MAP was associated with a
By week 4 after lentivirus delivery, lenti-ACE2 SHRs showed a 17 mm Hg decrease in MAP (141 ± 4 mm Hg in lenti-GFP versus 124 ± 5 mm Hg in lenti-ACE2) and a 19 bpm decrease in HR (304 ± 7 bpm in lenti-GFP versus 285 ± 5 bpm in lenti-ACE2) compared with GFP controls (n = 6 in each group). In contrast, lenti-ACE2 showed no effects on MAP and HR in WKY rats. In addition, no decrease in MAP and HR was observed in SHRs that were injected with lenti-ACE2 in the trigeminal nucleus in week 4 after the injection (143 ± 4 mm Hg; 298 ± 8 bpm; n = 5).

**Figure 2.** ACE2 protein levels in the RVLM of WKY rats and SHRs. A, Representative autoradiogram of ACE2 protein levels in the RVLM: Western blot analysis was used to measure ACE2 levels from 20 μg of total cell lysate isolated from the RVLM punches as described in the Methods section. Data were normalized using α-tubulin. B, Quantitation of the ACE2 protein band. *P < 0.05 vs WKY rats. Data were mean ± SEM (n = 4 in each strain).

**Discussion**

This study demonstrates that a persistent overexpression of ACE2 in the RVLM causes significant attenuation of high BP in the SHR. This, coupled with the observation that intrinsic ACE2 expression is decreased in the SHR RVLM, suggests the importance of this enzyme in neural-cardiovascular control and supports our view that ACE2 gene transfer is able to compensate for intrinsic ACE2 deficiency.

ACE2 immunoreactivity was diffusely distributed in the medulla oblongata, including the RVLM. The staining was primarily localized in the neuronal cell body but was not restricted to it, because axonal processes of some neurons were also stained. This distribution was consistent with the distribution of ACE2 in mouse brain reported by Doobay et al. They demonstrated the presence of ACE2 immunoreactivity in neurons of subfornical organ, the paraventricular nucleus, the area postrema, the dorsal motor nucleus of the vagus, the nucleus tractus solitarius (NTS), the nucleus ambiguus, and the RVLM. Localization of ACE2 in these discrete cardiovascular-regulatory nuclei was our initial indication that this enzyme may play a critical role in BP control and hypertension.

We decided to focus our attention on the RVLM for this investigation because of this area’s role as a final relay point before transmission of brain RAS-generated signals to the peripheral system. In addition, studies have shown an overactive RAS and increased AT1 receptors in the RVLM of the SHR. Pressor responses to microinjection of Ang II in the SHR RVLM were found to be greater than in the WKY rats. Furthermore, administration of valsartan into the SHR RVLM lowers high BP. Consistent with the hypothesis, we found that ACE2 levels were decreased in the SHR RVLM compared with its WKY rat control. In addition, overexpression of this enzyme by a lentiviral vector-mediated gene transfer system resulted in a significant attenuation of high BP and HR exclusively in the SHR. There are few interesting aspects...
of these observations: there seems to be a slow onset of the antihypertensive response, although lentiviral vector-mediated transgene expression has been shown to begin as early as 3 days and reaches robust levels within 1 week in an in vivo situation.\textsuperscript{17} In this study, transgenic ACE2 expression can be observed within a week. The reason for this discrepancy in slow onset of effects in BP and HR is not understood at the present time. It is tempting us to suggest that initially compensatory physiological mechanisms may be in play to counteract the effect of ACE2 in restoring baseline BP in the SHR. This proposal is supported by other studies.\textsuperscript{23,24} However, other possibilities cannot be ruled out at the present time. For example, it is quite possible that ACE2 transgene expression needs to reach a critical level for it to effectively influence Ang II and Ang-(1-7) levels. Next, although ACE2 overexpression reduces high BP and HR in the SHR, it has little effect on WKY rats. This observation is consistent with the lack of effect of Ang II type 1 receptor blockers on the BP of normotensive rats. It has been demonstrated that Ang II type 1 receptor blockers administered directly into the RVLM or via intravenous route have profound effects on the BP of hypertensive but not normotensive rats.\textsuperscript{4,5} These studies have led to the suggestion that the contribution of the RAS and ACE2 may be less significant in normal cardiovascular control because of the presence of redundant regulatory mechanisms and come into play only in pathological condition, such as hypertension and hypotension. Third, ACE2 overexpression, although it attenuates high BP to a significant degree, does not reduce BP to a normotensive level. This may be largely because of the fact that an overexpressed level of ACE2 in the RVLM of SHRs was still significantly lower than that of the WKY rat. Furthermore, because hypertension is a multigenetic disease, correction of > 1 gene would be required to accomplish normal BP levels. In addition, the role of other cardio regulatory brain areas (ie, subfornical organ, paraventricular nucleus, and NTS) in coordinating ACE2-dependent and -independent pathophysiological signaling and inputs to the RVLM cannot be minimized. Nonetheless, our study does implicate that the RVLM ACE2 is involved in hypertension in this rat model.

Finally, an important question remains regarding the mechanism of action of ACE2 in the RVLM. Because ACE2 is a key RAS enzyme proposed to balance levels of Ang II and Ang-(1-7), it is quite possible that ACE2 overexpression tips this balance toward Ang-(1-7). As a result, less Ang II is available to produce its hypertensive effects. Although conclusive evidence is lacking at the present time, our preliminary in vitro data support this proposal. They show that

\begin{figure}
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\includegraphics[width=\textwidth]{figure4}
\caption{ACE2 protein levels in the RVLM after ACE2 gene transfer. A, Representative autoradiogram of ACE2 protein levels in the RVLM: Western blot analysis was used to measure ACE2 protein levels from the RVLM punches of noninjected WKY rats and SHRs and SHRs 6 weeks after lenti-ACE2 injection. Data were normalized using \( \alpha \)-tubulin. B, Quantitation of the ACE2 protein band. Data were mean±SEM (\( n=4 \) in each group).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Effect of lenti-ACE2 on MAP and HR in WKY rats and SHRs. Lenti-GFP or lenti-ACE2 was injected into the bilateral RVLM of WKY rats (A) and SHRs (B), and MAP (left) and HR (right) were recorded in a conscious state using radioteremetry as described in the Methods section. Lenti-ACE2–injected SHRs showed a time-dependent decrease in MAP and HR. Data are mean±SEM (\( n=4 \) per group in WKY rats and \( n=6 \) per group in SHRs) *\( P<0.05 \) vs GFP control groups.}
\end{figure}
Ang-(1-7) has no significant effect on neuronal activity (unpublished data), indicating that the beneficial effects of ACE2 may not be as a result of generation of Ang-(1-7) but rather be a result of its ability to reduce Ang II levels. However, this view should be considered with caution, because in vitro studies may not be representative of the in vivo situation. A detailed analysis of Ang II type 1 receptor, Ang II, Ang-(1-7), and Mas must wait for the appropriate mechanism. In summary, our observations demonstrate that ACE2 levels are decreased in the SHR RVLM and that its overexpression produces significant antihypertensive effects on a long-term basis.

Perspectives

Brain RAS is relevant in the control of cardiovascular functions, and its hyperactivity is linked to hypertension. Thus, it is imperative to control this hyperactivity if neurogenic hypertension is to be successfully treated. Our studies are relevant in this respect, because they show, for the first time, that overexpression of ACE2 reduces high BP on a long-term basis. Use of a lentiviral vector enables us to carry out these chronic experiments and provide proof of concept that ACE2 in the RVLM is a therapeutically relevant target. This is coupled with the fact that novel lentiviral vectors are being developed that can facilitate retrograde transport of transgenes into distinct brain areas enabling easy access to relevant brain regions. Thus, we anticipate that, in the near future, one would be able to deliver lenti-ACE2 into appropriate peripheral locations that can be put into the RVLM to increase ACE2 expression to exert its beneficial effect in the control of neurogenic hypertension.

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Disclosures

None.

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