Overexpression of angiotensin-converting enzyme 2 attenuates tonically active glutamatergic input to the rostral ventrolateral medulla in hypertensive rats

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Wang YK, Shen D, Hao Q, Yu Q, Wu ZT, Deng Y, Chen YF, Yuan WJ, Hu QK, Su DF, Wang WZ. Overexpression of angiotensin-converting enzyme 2 attenuates tonically active glutamatergic input to the rostral ventrolateral medulla in hypertensive rats. Am J Physiol Heart Circ Physiol 307: H182–H190, 2014. First published May 16, 2014; doi:10.1152/ajpheart.00518.2013.—The rostral ventrolateral medulla (RVLM) plays a key role in cardiovascular regulation. It has been reported that tonically active glutamatergic input to the RVLM is increased in hypertensive rats, whereas angiotensin-converting enzyme 2 (ACE2) in the brain has been suggested to be beneficial to hypertension. This study was designed to determine the effect of ACE2 gene transfer into the RVLM on tonically active glutamatergic input in spontaneously hypertensive rats (SHRs). Lentiviral particles containing enhanced green fluorescent protein (lenti-GFP) or ACE2 (lenti-ACE2) were injected bilaterally into the RVLM. Both protein expression and activity of ACE2 in the RVLM were increased in SHRs after overexpression of ACE2. A significant reduction in blood pressure and heart rate in SHRs was observed 6 wk after lenti-ACE2 injected into the RVLM. The concentration of glutamate in microdialysis fluid from the RVLM was significantly reduced by an average of 61% in SHRs with lenti-ACE2 compared with lenti-GFP. ACE2 overexpression significantly attenuated the decrease in blood pressure and renal sympathetic nerve activity evoked by bilateral injection of the glutamate receptor antagonist kynurenic acid (2.7 nmol in 100 nl) into the RVLM in SHRs. Therefore, we suggest that ACE2 overexpression in the RVLM attenuates the enhanced tonically active glutamatergic input in SHRs, which may be an important mechanism underlying the beneficial effect of central ACE2 to hypertension.

It is well known that the rostral ventrolateral medulla (RVLM) is a key region for the central control of sympathetic outflow and plays a critical role in maintaining resting blood pressure (BP) and sympathetic tone (5, 20). ANG II, a main physiologically active effector peptide of the renin-angiotensin system (RAS), exerts its actions mainly by acting on the ANG II type 1 receptor (AT1R), thus contributing to cardiovascular regulation (1). ANG II signals, including AT1R expression and its sensitivity in the RVLM, are upregulated in hypertensive models, suggesting that increased ANG II signaling in the RVLM is critical in the development and maintenance of hypertension (11, 21, 33). Angiotensin-converting enzyme 2 (ACE2) is a member of the RAS and catalyzes the formation of ANG-(1–7) from ANG II and ANG-(1–9) (45). ANG-(1–7), a major product of ACE2, exerts opposite effects (e.g., vasodilation and antiproliferation) to ANG II, indicating that ACE2 would be a novel therapeutic target for hypertension (8, 26). In the brain, ACE2 is widely expressed in cardiovascular regulatory regions (10, 53). Furthermore, it has been demonstrated that ACE2 expression and activity in the RVLM are decreased in spontaneously hypertensive rats (SHRs), whereas ACE2 gene transfer into the brain, including the RVLM, is beneficial to hypertension (14, 41, 55). These observations suggest that central ACE2 plays an important role in the neural control of BP and sympathetic outflow in hypertension. However, the exact mechanism by which central ACE2 is beneficial to hypertension remains to be determined.

Glutamatergic synaptic transmission plays an important role in maintaining the resting activity of RVLM vasomotor neurons, thus acting as a driving element in keeping basal BP and sympathetic tone (5, 6, 20). Kubo et al. (28) reported that the content of glutamate in the RVLM is increased in SHRs compared with normotensive control rats [Wistar-Kyoto (WKY) rats]. Microinjection of the glutamate receptor (GluR) antagonist kynurenic acid (KYN) into the RVLM produces a significant fall in resting BP in hypertensive rats but not in normotensive rats (22, 23). We have also previously reported that enhanced tonic glutamatergic input to the RVLM contributes to hyperactivity of RVLM vasomotor neurons in rats with chronic heart failure, a model of hyperactive brain RAS (49). These observations suggest that the tonically active glutamatergic input to the RVLM is enhanced in hypertension (22, 23, 43). Interestingly, previous evidence has indicated a possible interaction between the RAS and glutamatergic transmission in cardiovascular regions (7, 11); for example, blockade of AT1Rs in the RVLM antagonizes the effects of glutamate (2). Chronic AT1R blockade in the paraventricular nucleus (PVN) leads to a significant reduction in N-methyl-D-aspartate (NMDA) receptor expression in rats with chronic heart failure (27). Although it has been demonstrated that ACE2 overexpression in the RVLM leads to a long-term decrease in BP in SHRs (55), there is no direct evidence showing the relationship between ACE2 and tonically active glutamatergic input in the RVLM. Therefore, the major aim of the present study was to determine if the enhanced tonically active glutamatergic input to the RVLM is reduced by ACE2 overexpression in hypertension.

METHODS

Animals. Twelve-week-old male SHRs and WKY rats were purchased from Sino-British SIPPR/BK Laboratory Animal (Shanghai, China; and were housed in a constant environment of 22 ± 1°C and a 12:12-h light-dark cycle, with food (alimentary paste, MF, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and water available ad libitum. Animals were acclimated for 1 wk before experiments.
China). Rats were housed in a temperature-controlled room and kept on a 12:12-h light-dark cycle with free access to water. All procedures in this study were approved by the Institutional Animal Care and Use Committee of Second Military Medical University.

**Cloning of human ACE2 in lentiviral vector and production of lenti-ACE2.** The construction and production of the lentiviral vector were by Invabio (Shanghai Innovation Biotechnology, Shanghai, China), as previously described (19). Human (h)ACE2 cDNA (Accession No. NM_021804) was used as a template for PCR amplification with the use of the following primer sequences: sense 5'-ATGCTGCGCGCCGACTGCACAACTCG-3' and antisense 5'-TTACGAGGTTTCTCTGTTGCACTTT-3'. The hACE2 amplification product was cloned into lentivirus cloning vector pLenO-DCE (Invabio). The pLenO-DCE vector used in these experiments carried a green fluorescent protein (GFP) reporter gene. The presence of an internal ribosome entry site element permits the expression of two gene products (GFP and ACE2) from a single promotor. Note that the control construct (lenti-GFP) contained all sequence elements except for the therapeutic transgene (hACE2). Briefly, lentivirus packaging helper plasmids such as pRSV-Rev, pMDLG/pRRE, and pMD2.G were used to facilitate the transfection of the therapeutic transgene plasmid (pLenO-DCE-ACE2) in 293T cells. Virus-containing media of 293T cells were then harvested at 48h posttransfection, concentrated by ultrafiltration and ultracentrifugation, aliquoted, and stored at −80°C. The number of transducing units, defined as an infectious particle, was determined by estimating the number of GFP-positive cells by FACS analysis in 293T cell suspensions, and the final infectious titer was ~5 × 10^6 transducing units/ml.

**Gene delivery into the RVLM.** Rats were anesthetized with inhaled isofluorane (3%) and placed in a stereotaxic instrument (Shanghai Alcott Biotech). After the skull was exposed through an incision on the midline of the scalp, a small hole (2.0 mm lateral to the midline) was made under specific pathogen-free conditions. Rats were individually anesthetized with inhaled isofluorane (3%) and placed in a stereotaxic instrument (Shanghai Alcott Biotech). After viral administration, rats were housed individually under specific pathogen-free conditions. Rats were divided into four groups (WKY, SHR, SHR-GFP, and SHR-ACE2) in this study.

**Measurement of BP by a tail-cuff system in conscious rats.** BP and heart rate (HR) in conscious rats were measured noninvasively using a computerized noninvasive tail-cuff system (ALC-NIBP, Shanghai Alcott Biotech), as previously described (56). Four parameters were simultaneously monitored: systolic BP, diastolic BP, mean arterial pressure (MAP), and HR. Conscious rats were warmed to an ambient temperature of 30°C to help vasodilate the tail artery by placing them comfortably in the chambers during this process. Using a noninvasive tail-cuff system (WPI), the injection speed was controlled by an ultramicropump (WPI). After viral administration, rats were housed individually under specific pathogen-free conditions. Rats were divided into four groups (WKY, SHR, SHR-GFP, and SHR-ACE2) in this study.

**Measurement of ACE2 activity.** Rats were euthanized with an overdose of pentobarbital sodium (200 mg/kg) and perfused through the aorta with 0.9% NaCl solution and 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4°C. Brain blocks were transferred to 20% sucrose solution. Sections of 20 μm thickness were cut in a cryostat and floated in 0.01 M PBS (pH 7.4). The floating sections were washed three times for 5 min in PBS and preincubated in 10% BSA in PBS for 30 min. All incubations and reactions were separated by three 5-min washes in 0.1 M PBS. Finally, sections were then mounted on slides and embedded in antifade mounting medium. The GFP staining (excitation: 488 nm and emission: 530 nm) was detected with a Olympus digital camera DP72 system. The GFP staining (excitation: 488 nm and emission: 530 nm) was detected with a Olympus digital camera DP72 system. The GFP staining (excitation: 488 nm and emission: 530 nm) was detected with a Olympus digital camera DP72 system.

**In vivo brain microdialysis.** In vivo brain microdialysis was carried out to measure glutamate content in the extracellular fluid in the RVLM, as previously described (37, 56). After rats had been anesthetized with an overdose of pentobarbital sodium (200 mg/kg) and perfused through the aorta with 0.9% NaCl solution and 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4°C. Brain blocks were transferred to 20% sucrose solution. Sections of 20 μm thickness were cut in a cryostat and floated in 0.01 M PBS (pH 7.4). The floating sections were washed three times for 5 min in PBS and preincubated in 10% BSA in PBS for 30 min. All incubations and reactions were separated by three 5-min washes in 0.1 M PBS. Finally, sections were then mounted on slides and embedded in antifade mounting medium. The GFP staining (excitation: 488 nm and emission: 530 nm) was detected with a Olympus digital camera DP72 system.

**Activity following the manufacturer’s instructions.** The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions. The assay of ACE2 activity followed the manufacturer’s instructions.
HPLC. As previously described (37, 56, 57), HPLC (model 582 pump, ESA) with electrochemical detection (model 5300, ESA) was used to detect the glutamate concentration in dialysate samples from the RVLM and 24-h urinary excretion of norepinephrine (NE).

The derivatization of O-phthalaldehyde/β-mercaptoethanol was used to determine the amino acid glutamate, as described in our previous study (56). Glutamate was separated by HPLC with a reverse-phase C18 column (Shiseido Capcell Pak 75 × 3 mm, 3 μm C18, P/ N 88-90816) and further quantified with electrochemical detection. The mobile phase was composed of 100 mM di-sodium hydrogen phosphate anhydrous, 22% methanol, and 3.5% acetonitrile at pH 6.75, and the flow rate was 0.60 ml/min. The detect channel potentials were set at +150 and +550 mV. The HPLC system was controlled, and data were acquired, processed, and analyzed using Coulochem software. The analyte glutamate was identified according to the authentic standard based on its retention time and peak height ratio. The content of glutamate was quantified by a comparison of the area with the standard.

Measurement of urinary NE was based on our previous study (56). Briefly, 24-h urinary samples were collected after rats were placed in metabolism cages for 24 h and acidified with glacial acetic acid in 15-ml centrifuge tubes, which were emplaced in crushed ice. Dihydroxybenzylamine (Sigma) was used as the internal standard. NE was absorbed onto acid-washed alumina with 3 mol/l Tris(hydroxymethyl) aminomethane buffer at pH 8.6 in 2% EDTA. The alumina was then washed 3 times with 3 ml of distilled water. NE was extracted with 400 μl of 0.2 M glacial acetic acid with 5 min of shaking and a final 30-min settlement. The supernatant (40 μl) was injected into the HPLC column [reverse phase, ESA, 150 × 3.2 mm, 3 μm C18 (P/N 70-0636)], and NE was eluted with the mobile phase (80 mM citric acid monohydrate, 73.4 mM citric acid trisodium salt, 0.12 mM 1-octanumonic acid sodium salt, and 0.1 mM EDTA adjust to pH 4.3 with phosphoric acid). The flow rate was set at 0.5 ml/min.

Intracerebroventricular infusion. To determine the role of the Mas receptor in mediating the effect of ACE2 overexpression on the release of glutamate in the RVLM, intracerebroventricular (fourth ventricle) infusion of the Mas receptor antagonist A779 was performed on the fifth wk after lenti-ACE2 injected into the RVLM of SHRs. Rats in the fifth week of ACE2 overexpression were anesthetized with isoflurane (3%) and fixed on the stereotaxic frame. The atlantooccipital membrane was exposed and punctured with a needle. The cannula connect to the osmotic minipump capsule (0.5 μl/h, 1000D, ALZET) was implanted into the fourth ventricle along the pinhole and fixed with biological glue. After 1 wk of continuous infusion of A779 (1 nmol/day) or vehicle (artificial cerebrospinal fluid), rats were subsequently subjected to in vivo brain microdialysis.

Responses of BP, HR, and renal sympathetic nerve activity to acute RVLM microinjections. Acute in vivo experiments were performed to detect the cardiovascular sensitivity [BP, HR, and renal sympathetic nerve activity (RSNA)] to injection of GluR antagonist into the RVLM. Surgical procedures, recording of RSNA, and RVLM microinjections were carried out as we have previously described (36, 37, 49, 50, 56). Briefly, rats were anesthetized with urethane (800 mg/kg ip) and α-chloralose (40 mg/kg ip), and the trachea was cannulated to facilitate mechanical respiration with a ventilator (SAR-830, CWE). The concentration of end-tidal CO2 was monitored and kept at the level of ~4% by a CO2 analyzer (Capstar 100, CWE). The right forelimb was catheterized for BP measurement by PowerLab (SSP, AD Instruments). MAP and HR were derived from the BP pulse. The degree of anesthesia was monitored by the BP response to a nociceptive stimulus paw pinch. An adequate depth of anesthesia was defined by observing a small change (<20%) of BP in response to a paw pinch at intervals throughout the experiment, as previously described (13). Additional doses of anesthetic (10 mg/kg iv α-chloralose) were administrated as required. The rat was placed in a stereotactic frame with the head fixed horizontally, and the dorsal surface of the medulla was surgically exposed. The left renal sympathetic nerves were exposed, identified, dissected free of the surrounding connective tissue, and placed on a pair of platinum-iridium recording electrodes. The distal terminal of the renal nerve was cut to avoid afferent activity. RSNA was amplified (band pass: 100–3,000 Hz) and monitored by the PowerLab system. The preinjection level (control) of RSNA was taken as 100% from the absolute value after the noise level was subtracted. Body temperature was kept at 37°C by a temperature controller.

Microinjections in the RVLM were made from a three-barrel micropipette. The coordinates for injections into the RVLM were 2.0–2.5 mm rostral to the caudal tip of the area postrema, 2.0–2.2 mm lateral to the medline, and 3.0–3.2 mm below the dorsal surface of the brain stem. The RVLM injection (100 nl) was made over a period of 5–10 s by a pressure injector (PV820, WPI). A pressor response (at least 20 mmHg) evoked by microinjection of L-glutamate (2 nmol) was used to chemically identify the RVLM. Based on previous studies (22–24, 56), a 2.7 nmol (100 nl) dose of KYN (Sigma) was chosen to block ionotropic GluRs at least 30 min after a functional pressor site in the RVLM was identified. The interval of bilateral injections was within 2 min. Continuous recordings of BP, HR, and RSNA were at least 60 min after bilateral injection of KYN into the RVLM. At the end of the experiment, the rat was euthanized with an overdose of pentobarbital sodium. The maximum of RSNA occurred 1–2 min after the rat was euthanized. As previously described (47), the baseline RSNA value of individual rat was taken as a percentage of the maximum RSNA. Injection sites were also marked by injection of dye blue, and the rat was perfused with 10% formaldehyde solution (100 ml) intracardially. The brain stem was then quickly removed and fixed in 10% buffered formalin. Frozen 50-μm coronal sections were made using a freezing microtome and mounted on slides. Injection sites were checked according to the standard atlas of Paxinos and Watson (35).

Data analysis. Data are presented as means ± SE. Statistical comparisons between experimental groups were made by a Student’s t-test or one-way ANOVA followed by a Newman-Keuls post hoc test. Differences were considered to be significant for P values of <0.05.

RESULTS

Efficacy of lenti-ACE2 gene transfer to the RVLM. Figure 1A shows that GFP expression was restricted to the area of the RVLM. We confirmed that the level of ACE2 expression in the RVLM was significantly decreased in untreated SHRs compared with untreated WKY rats. We found that ACE2 was expressed in both neurons and fibers. We further observed an average of an approximately twofold increase (P < 0.05) in ACE2 expression in SHRs 4 wk after lenti-ACE2 injection into the RVLM compared with lenti-GFP injection (Fig. 1B). Similar to ACE2 protein expression, ACE2 activity in the RVLM was also found to be decreased in SHRs compared with WKY rats. Overexpression of ACE2 produced a significant (P < 0.05) increase of 69% in ACE2 activity in SHRs (Fig. 1C).

Effect of ACE2 overexpression in the RVLM on BP, HR, and 24-h urinary excretion of NE. As shown in Fig. 2, levels of BP and HR began to decrease in SHRs 3 wk after lenti-ACE2 injection compared with lenti-GFP injection. This reduction in BP and HR persisted until the time (6 wk) of termination of the experiment. However, levels of BP and HR in lenti-ACE2-transfected SHRs were still higher than those in untreated WKY rats. In addition, lenti-ACE2 injected into the RVLM had no effect on baseline BP and HR in WKY rats (Fig. 2B). Compared with WKY rats, SHRs showed a significant increase in 24-h urinary excretion of NE. However, 24-h urinary excretion of NE was not significantly different between the lenti-ACE2 and control groups.
tion of NE was significantly reduced in the SHR-ACE2 group compared with the SHR-GFP group (0.36 ± 0.06 vs. 0.59 ± 0.03 μg, n = 5, P < 0.05; Fig. 2C).

Effect of ACE2 overexpression on the release of glutamate in the RVLM. As shown in Fig. 3A, the concentration of glutamate in the microdialysis fluid of the RVLM was significantly higher in untreated SHRs compared with untreated WKY rats (1,476 ± 137 vs. 280 ± 83 μg/l, n = 5, P < 0.05). The content of glutamate was significantly reduced (1,586 ± 165 vs. 622 ± 70 μg/l, P < 0.05) in SHRs 6 wk after RVLM injection of lenti-ACE2 compared with lenti-GFP, but it was still higher than in WKY rats. Furthermore, the ACE2-induced reduction in glutamate release in SHRs was significantly blunted after treatment with intracerebroventricular infusion of the Mas receptor antagonist A779 (1 nmol/day, 1 wk) in the fifth week of lenti-ACE2 injection into the RVLM of SHRs. Moreover, we also observed a relationship between the level of ACE2 protein expression and glutamate release at different time points (baseline, second week, fourth week, and sixth week) after lenti-ACE2 injected into the RVLM of SHRs (Fig. 3C). These results suggest that a gradual decrease in glutamate content was accompanied by a gradual increase in ACE2 expression after lenti-ACE2 injections in SHRs.

Effect of ACE2 overexpression on the decreases in BP, HR, and RSNA evoked by blockade of GluRs in the RVLM. As shown in Table 1, baseline BP, HR, and RSNA in anaesthetized rats were reduced in SHRs 6 wk after ACE2 overexpression in the RVLM. Figure 4 shows original tracings of BP, HR, and RSNA in response to microinjection of the GluR antagonist KYN (2.7 nmol) into the RVLM. Bilateral injection of KYN into the RVLM produced a significant decrease in BP, HR, and RSNA in untreated SHRs but not in untreated WKY rats. However, these decreases in BP (−22.7 ± 1.8 vs. −42.4 ± 3.7 mmHg), HR (−21.9 ± 4.1 vs. −41.1 ± 3.5 beats/min), and RSNA (−11.6 ± 0.9 vs. −20.6 ± 2.6%) induced by KYN in the RVLM were significantly lower (n = 5, P < 0.05) in SHRs that received lenti-ACE2 injection compared with lenti-GFP injection (Fig. 5).

Effects of ACE2 overexpression on protein levels of AT₁R, Mas receptor, and GluR expression in the RVLM of SHRs. Figure 6A shows the effect of ACE2 overexpression on levels of AT₁R and Mas protein expression in the RVLM of SHRs. A reduction in AT₁R expression was found in the RVLM of lenti-ACE2-injected SHRs compared with lenti-ACE2-injected SHRs, whereas the Mas receptor was upregulated in SHRs with lenti-ACE2 treatment. In addition, ACE2 overexpression produced a reduction of 49% and 50% in protein levels of
NMDA receptor 1 and GluR5/6/7 in the RVLM of SHRs, respectively (Fig. 6B).

**DISCUSSION**

The major finding of the present study are as follows: 1) overexpression of ACE2 in the RVLM produced a long-term decrease in BP in SHRs; 2) ACE2 overexpression significantly reduced the release of glutamate in the RVLM of SHRs; and 3) ACE2 overexpression significantly blunted the response of BP, HR, and RSNA to blockade of GluRs in the RVLM of SHRs. On the basis of these results, we conclude that ACE2 overexpression effectively attenuates the enhanced tonically active glutamatergic input to the RVLM, which may be an important contributor to the beneficial effects of central ACE2 on hypertension.

It has been previously reported that lentiviral vector-mediated transgene expression begins as early as 3 days postinfection. However, in the present study, levels of BP and HR began by 10.220.32.247 on September 22, 2017 http://ajpheart.physiology.org/ Downloaded from

![Fig. 2. Effect of ACE2 overexpression in the RVLM on blood pressure (BP), heart rate (HR), and 24-h urinary excretion of norepinephrine (NE). A: time course of mean arterial pressure (MAP; left) and HR (right) measured by tail-cuff system in the WKY, SHR, SHR-GFP, and SHR-ACE2 groups. A time-dependent reduction in MAP and HR was induced by lenti-ACE2 injected into the RVLM in SHRs compared with lenti-GFP injection. Base, baseline. n = 15 rats/group. *P < 0.05 vs. the SHR-GFP group. B: MAP in WKY rats injected with lenti-GFP (WKY-GFP group) and lenti-ACE2 (WKY-ACE2 group). C: effect of ACE2 overexpression on 24-h urinary excretion of NE. n = 5 rats/group. *P < 0.05 vs. the WKY group; #P < 0.05 vs. the SHR-GFP group.](http://ajpheart.physiology.org/)

![Fig. 3. Effect of ACE2 overexpression in the RVLM on glutamate concentration. A: content of glutamate in the dialysis fluid from the RVLM from the WKY, SHR, SHR-ACE2, SHR-GFP groups with or without pretreatment with intracerebroventricular infusion (1 wk) of the Mas receptor antagonist A779. n = 5 rats/group. *P < 0.05 vs. the WKY group; #P < 0.05 vs. the SHR-GFP group; $P < 0.05 vs. the SHR-ACE2 group + artificial cerebrospinal fluid (aCSF). B: time course of ACE2 expression levels (top) and glutamate contents (bottom) at baseline and on the second, fourth, and sixth wk after ACE2 gene transfer into the RVLM of SHRs. n = 4 rats/group. *P < 0.05 vs. baseline; #P < 0.05 vs. the value in the fourth wk.](http://ajpheart.physiology.org/)
to decrease 3 wk after lenti-ACE2 injected into the RVLM. This observation is agreement with a previous study by Yamazato et al. (55), in which the authors explain the initially compensatory physiological mechanisms responsible to counteract the effect of ACE2 in restoring BP in SHRs. We found that BP and HR were kept at lower levels on the fourth to sixth week in SHRs with lenti-ACE2 treatment. Moreover, ACE2 activity and protein expression in the RVLM were significantly increased 4 wk after ACE2 gene transfer. The presented data show that maximum ACE2 expression is reached at 4 wk after ACE2 gene delivery in SHRs (Fig. 3B). Moreover, the level of ACE2 expression is still higher at 6 wk after gene delivery compared with control. In addition, SHRs 6 wk after lenti-ACE2 injection into the RVLM showed a significant decrease in 24-h urinary excretion of NE and basal RSNA (percentage of maximum), indicating that ACE2 overexpression in the RVLM reduces sympathetic activity in SHRs. It was notable that ACE2 overexpression in the RVLM of SHRs decreased the BP level, which was still higher than the BP level of WKY rats. However, 24-h urinary excretion of NE in SHRs with ACE2 overexpression was completely restored to its level in WKY rats. In addition to sympathetic activity, alternations in vagal nerve activity also have an effect on BP level. Lenti-ACE2 injections were located in the sympathetic center of the RVLM but not in the vagal center nucleus ambiguous. Therefore, it is possible that the reduction of sympathetic activity by ACE2 in the RVLM was not sufficient to return the BP level to that of WKY rats. Based on the above evidence, SHRs received lenti-ACE2 or lenti-GFP injections, and results were analyzed after 6 wk to determine the interaction between ACE2 and glutamatergic input at the level of the RVLM. Similar to a previous study (55), we also revealed (Fig. 2B) that ACE2 overexpression in the RVLM did not alter the BP level in WKY rats, suggesting that ACE2 may not contribute to maintaining the resting BP level under normotensive conditions. In this study, untreated WKY rats and SHRs were used to as controls for SHRs with lenti-ACE2 or lenti-GFP gene transfer. In addition, we also note two limitations in our approach of ACE2 gene delivery into the RVLM. The area of the RVLM for ACE2 gene delivery was not functionally identified by a pressor response to L-glutamate. However, according to the distribution of GFP immunostaining, we confirmed that the distribution of gene delivery was accurately located within the RVLM1 area. Second, a tail-cuff system was used to

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<tr>
<th>Number of rats</th>
<th>WKY Group</th>
<th>SHR Group</th>
<th>SHR-GFP Group</th>
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<tr>
<td>MAP, mmHg</td>
<td>120 ± 4</td>
<td>172 ± 5*</td>
<td>178 ± 4</td>
<td>154 ± 3*†</td>
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<td>HR, beats/min</td>
<td>383 ± 8</td>
<td>387 ± 12</td>
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<td>46.4 ± 4.4*</td>
<td>47.3 ± 3.7</td>
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Data are means ± SE. Blood pressure was measured by catheterizing the femoral artery. MAP, mean arterial pressure; HR, heart rate; RSNA, renal sympathetic nerve activity. Rats were divided into the following four groups: untreated Wistar-Kyoto (WKY) rats (WKY group), untreated spontaneously hypertensive rats (SHRs; SHR group), lenti-green fluorescent protein (GFP)-injected SHRs (SHR-GFP group), and lenti-angiotensin-converting enzyme 2 (ACE2)-injected SHRs (SHR-ACE2 group). *P < 0.05 vs. the WKY group; †P < 0.05 vs. the SHR-GFP group.
monitor BP in conscious rats with gene transfer. This tail-cuff method is a form of restraint and, as such, may stress the animal and alter the level of BP. Moreover, the RAS in the RVLM may be involved in the pressor response to stress (3). In this work, however, we found a similar BP level obtained in anesthetized rats by catheterizing the femoral artery compared with in conscious rats by the tail-cuff method.

The main goal of the present study was to determine the effect of ACE2 overexpression on the tonically active glutamatergic inputs to the RVLM. Consistent with previous studies (22, 23), we also confirmed that tonically active glutamatergic input to the RVLM is enhanced in SHRs. Furthermore, we found that ACE2 overexpression in the RVLM in SHRs significantly reduced the release of glutamate but also significantly blunted the reduction in BP, HR, and RSNA evoked by microinjection of KYN into the RVLM. Moreover, the gradual decrease in glutamate content was accompanied by a gradual increase in ACE2 expression level after ACE2 gene transfer into the RVLM of SHRs, suggesting that this reduction in glutamate release is probably produced by ACE2 overexpression in SHRs. Consistent with previous studies (10, 55), we confirmed that ACE2 was expressed in both the cell body as well as nerve terminal fibers. Therefore, we suggest that ACE2 overexpression is capable of attenuating the enhanced tonically active glutamatergic inputs to the RVLM in SHRs. We also initially observed the changes of glutamate receptors in response to ACE2 overexpression in SHRs. We found that protein levels of NMDA receptor 1 (an ionotropic GluR subtype) and GluR5/6/7 (a non-NMDA receptor) in SHRs were downregulated by ACE2 overexpression. It is likely that ACE2 overexpression reduces the functional state of GluRs in SHRs. The level of NMDA receptor expression is different between WKY rats and SHRs (12), and SHRs are more sensitive to l-glutamate or NMDA in the RVLM (32, 44). However, it has been documented that injection of glutamate into the RVLM affects blood pressure to the same extent in both strains (39). The significance of the functional state of GluRs in the RVLM in mediating the beneficial effect of ACE2 on hypertension needs to be further determined.

Clearly, the results of the present study do not adequately address the question of how ACE2 gene transfer into the RVLM reduces tonically active glutamatergic input. ACE2 overexpression leads to an increase in the production of intrinsic ANG-(1–7) (45). Upregulation of the ACE2/ANG-(1–7)/Mas axis in the brain has been become a potential therapeutic strategy for the treatment of hypertension and chronic heart failure (8, 14, 53, 54). It has been reported central chronic application of ANG-(1–7) is capable of lowering high BP or restoring the blunted baroreflex sensitivity in these cardiovascular diseases (18, 25). In this study, we found that ACE2 overexpression significantly increased protein levels of the ANG-(1–7)-acting receptor Mas; this result is supported by a recent study (41) showing ACE2 overexpression in the PVN upregulated Mas receptor expression in ANG II-induced hypertension. Importantly, it has been suggested that ANG-(1–7) is capable of modulating the release of neurotransmitters (e.g., dopamine, GABA, and glutamate) via Mas receptors in the brain (42, 48). The present data have further demonstrated that the ACE2-mediated reduction in glutamate release in the RVLM is a form of restraint and, as such, may stress the animal and alter the level of BP. Moreover, the RAS in the RVLM may be involved in the pressor response to stress (3). In this work, however, we found a similar BP level obtained in anesthetized rats by catheterizing the femoral artery compared with in conscious rats by the tail-cuff method.
RVLM of SHRs was significantly blunted by pretreatment with blockade of the Mas receptor, suggesting that the ANG(1–7)/Mas axis contributes, at least partially, to the inhibitory effect of ACE2 on glutamate release in the RVLM. Interestingly, Wang et al. (48) reported that acute injection of exogenous ANG(1–7) into the RVLM produced an increase in BP accompanied by an increased release of glutamate in normotensive rats. However, the data from the present study and previous study (55) have demonstrated that a long-term increase in endogenous ANG(1–7) by ACE2 gene transfer into the RVLM significantly reduces BP in SHRs. Differential biological actions of Mas receptor activation by ANG(1–7) may be dependent on some conditions (e.g., acute vs. chronic, endogenous vs. exogenous, and the BP level of animals). The exact mechanisms underlying the effect of Mas receptor activation on central cardiovascular regulation need to be further defined.

On the other hand, recent evidence suggests a possible interaction between ACE2 and the ACE/ANG II/AT1R axis (52, 53). We found that protein expression of AT1Rs in the RVLM is downregulated in SHRs treated with ACE2 overexpression; this finding is agreement with previous studies showing that ACE2 overexpression in the PVN or subfornical organ downregulates AT1Rs in ANG II-induced hypertension (15, 41). It has also been reported that AT1Rs can be expressed in the presynaptic terminal (29). Thus, a downregulated ACE/ANG II/AT1R axis may be in play to result in the beneficial effects of ACE2 in the RVLM on hypertension. ANG II injected into the RVLM stimulates the release of glutamate (57). In addition to the RVLM, it has also been suggested that ANG II-mediated excitation of magnocellular PVN neurons is dependent on an increase in glutamatergic input (30). Furthermore, it has been suggested that increased ROS, which are mainly derived by ANG II, contribute to enhanced glutamatergic input to the RVLM in SHRs (34). On basis of the above observations, downregulation of the ANG II/AT1R axis may play an important role in mediating the effect of ACE2 overexpression on tonically active glutamatergic input to the RVLM in hypertension. However, the exact mechanism(s) by which alterations of AT1Rs and Mas receptors by ACE2 influence the release of glutamate needs to be further defined.

AT1Rs and Mas receptors are reported to be expressed in presynaptic terminals and involved in mediating the release of neurotransmitters (17, 31). Ca2+ influx is necessary for the release of neurotransmitters from presynaptic terminals (38). Interestingly, it has been suggested that ANG II increases Ca2+ influx (16, 46), whereas ANG-(1–7) may decrease Ca2+ influx, perhaps through triggering nitric oxide signaling (9, 40, 51). Therefore, it is likely that the ACE2-induced decrease in glutamate release in the RVLM of SHRs is associated with decreased Ca2+ influx, which may be dependent on functional states of the AT1R and Mas receptor. In addition, we also realize that future experiments may be necessary to further differentiate the ACE2 beneficial effect. For example, comparison of ACE2 beneficial effects with AT1R blockade alone or ANG(1–7) infusion alone will reveal more about the involvement of individual components.

In summary, the present results have shown that ACE2 gene transfer to the RVLM in SHRs attenuates the increased tonically active glutamatergic inputs to the RVLM in SHRs. Normalization of the enhanced glutamatergic input in RVLM neurons is an important mechanism underlying the beneficial effects of ACE2 on high BP and sympathetic hyperactivity in hypertension. The present work provides new information to extend our knowledge of the ACE2-induced adjustment of central autonomic networks for cardiovascular activity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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