Gene transfer of angiotensin-converting enzyme 2 in the nucleus tractus solitarius improves baroreceptor heart rate reflex in spontaneously hypertensive rats

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Abstract
The renin–angiotensin system (RAS) in the nucleus tractus solitarius (NTS) is an important modulator of the baroreceptor heart rate reflex. This study tested the hypothesis that angiotensin-converting enzyme 2 (ACE2) expression is decreased in the NTS of spontaneously hypertensive rats (SHRs) and that its gene transfer in this nucleus would lead to beneficial effects on baroreflex function since this enzyme is key in the regulation of the vasoprotective axis of the RAS. ACE2 protein levels and its activity were significantly decreased in the NTS of SHRs compared to normotensive Wistar-Kyoto (WKY) control rats. Rats instrumented with radio-telemetry transducers received NTS microinjection of either Lenti-ACE2 (Lentiviral vector-mediated gene transfer of ACE2) or lenti-GFP (green fluorescent protein). The ACE2 gene transfer into the NTS resulted in long-term overexpression of ACE2. This was associated with a 60% increase in heart rate baroreflex sensitivity in the lenti-ACE2 injected SHRs compared with the lenti-GFP injected control SHRs (0.27 ± 0.02 ms/mmHg in lenti-GFP rats vs. 0.44 ± 0.07 ms/mmHg in lenti-ACE2 rats). These observations demonstrate that ACE2 gene transfer overcomes its intrinsic decrease in the NTS of SHRs and improves baroreceptor heart rate reflex.

Keywords
Blood pressure, gene transfer, Lentiviral vector, NTS, Wistar-Kyoto rats

Introduction
Increasing evidence indicates that a hyperactive brain renin–angiotensin system (RAS) is critical in the development and maintenance of hypertension. The nucleus tractus solitarius (NTS) in the brainstem receives baroreceptor afferent inputs and has an important role in modulating baroreflex function. Significance of the NTS is further highlighted by observations that increases in NTS sensitivity to angiotensin (Ang) II and impaired baroreflex function are associated with hypertension. This conclusion is supported by observations that density of Ang II binding sites is increased in the NTS of hypertensive animals, and microinjection of Ang II into the NTS attenuates baroreflex sensitivity, whereas Ang II antagonists enhance baroreflex sensitivity. Thus, regulation of the RAS activity in the NTS is critical in long-term regulation of baroreflex function and neural control of blood pressure (BP).¹⁻³

Recent discovery of angiotensin-converting enzyme 2 (ACE2) provides a novel target for chronic regulation of the brain RAS with implications on long-term beneficial outcomes to the cardiovascular system.⁴⁻⁸ ACE2 has been implicated in maintaining the balance between the two axes of the RAS, i.e. the vasoconstrictor, proliferative and...
fibrotic axis (ACE-Ang II-AT1 receptor) and the vasoprotective axis consisting of ACE2, Ang-(1-7) and Mas receptor. As a result, it has been suggested that a decrease in intrinsic ACE2 would lead to hypertension and its related pathophysiology, whereas its overexpression or activation would be beneficial effects in these conditions. Evidence has shown that a decrease in ACE2 is associated with cardiac and renal diseases. Our observations have demonstrated that ACE2 overexpression attenuates development of hypertension-induced cardiac remodeling in the Ang II infusion rat model of hypertension. In addition, ACE2 gene transfer both lowers BP and improves cardiac pathology in spontaneously hypertensive rats (SHRs). Furthermore, overexpression of ACE2 in the rostral ventrolateral medulla (RVLM) partially restores its intrinsic decrease and reduces high BP in the SHR. Despite dramatic beneficial effects of ACE2 and the observation that ACE2 is present in various cardiovascular regulatory brain areas, the role of this enzyme in hypertension has not been fully delineated. These observations coupled with the importance of the NTS in central cardiovascular regulation led us to propose the following hypothesis: ACE2 expression is decreased in the NTS of hypertensive rats and its overexpression in this nucleus would lead to beneficial effects on baroreflex function. Thus, the current study was conducted to support or refute this hypothesis.

**Methods**

**Animals**

Six month-old male SHR and Wistar-Kyoto (WKY) rats were purchased from Harlan Sprague-Dawley, Inc. (Harlan Tekland, Madison, WI). Rats were housed individually and maintained in a 12:12 light–dark cycle in a climate-controlled room. Rats’ chow and water were provided ad libitum. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Florida.

**Real-time RT-PCR**

Using a Harris Micro-Punch (1.0 mm, Electron Microscopy Sciences, Hatfield, PA), both sides of the caudal NTS were punched out of a 1-mm-thick section from WKY (n = 4) and SHR (n = 4) rats, as previously described. Total RNA was extracted from the punches with RNaqueous-4RCP Kit (Ambion). cDNA samples (2 μl) of reverse transcription reactions were amplified by quantitative real-time PCR (qPCR) using primers and probes for ACE2 from Applied Biosystems (Cat# Mm01159013_m1), and an ABI PRISM 7900 HT Detection system. ACE2 mRNA levels were normalized to 18S RNA from the same samples.

**Western blot analysis**

The NTS punches were obtained from the medulla oblongata of WKY (n = 8) and SHR (n = 8) rats. Proteins were extracted from the punches and samples containing 20 μg of protein were run on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Following 1 h blocking with 5% milk in Tris-buffered saline-Tween (TBS-T), the membrane was probed with the rabbit polyclonal antibody to ACE2 (1:1000, GTX15348, GeneTex) in 1% BSA/TBS-T overnight. The membrane was washed three times for 10 min in TBS-T and incubated with anti-rabbit IgG-HRP-conjugated secondary antibody (1:5000) for 1 h. The membrane was incubated with chemiluminescent agent for 1 min and exposed to a film to visualize protein bands. Alpha-tubulin bands were analyzed in parallel and used as a loading control for normalization of the ACE2 protein levels.

**Measurement of ACE2 activity**

ACE2 activity measurement was carried out as previously illustrated. In brief, NTS punches (n = 4-8) were rinsed with PBS, pH 7.5, lysed in 100 μl of reaction buffer (1 mol/L NaCl, 75 mmol/L Tris, 0.5 mmol/L ZnCl2, pH 7.5), sonicated and centrifuged at 20,800 g for 5 min. Cell supernatants containing 70 μg of protein were incubated with 100 μmol/L of fluorogenic peptide substrate VI (FPSVI; R&D Systems, Minneapolis, MN) and 10 μmol/L captopril in a final volume of 100 μl of reaction buffer at room temperature. Increase in the fluorescence at 405 nm was monitored using a SpectraMax Gemini EM Fluorescence Reader (Molecular Devices, Sunnyvale, CA) to measure total ACE2 activity. Data are presented as substrate FPSVI converted to product per unit of time and are normalized to total protein.

**Production of lenti-ACE2 viral particles**

Lentiviral particles containing enhanced green fluorescent protein (EF1α-IRE5-EGFP, lenti-GFP) or murine ACE2 (EF1α-ACE2-IRE5-EGFP, lenti-ACE2) were prepared as described previously. Viral medium containing lenti-GFP or lenti-ACE2 was collected, concentrated and titered. Concentration of viral particles was determined with the use of HIV-1 p24 antigen ELISA assay (Beckman Coulter, Fullerton, CA) following the manufacturer’s instructions. Efficacy of lenti-ACE2 in producing active ACE2 enzyme has been previously established.

**Abdominal aorta cannulation**

Rats were anesthetized with inhaled isoflurane (3%) in all surgical procedures. Radiotelemetric pressure transducers (Data Sciences international, Arden Hills, MN) consisting of a fluid-filled catheter attached to a PA-C40 transmitter were implanted into the abdominal aorta as described previously. Before implantation, the aorta was clamped proximally and the
catheter inserted and secured with medical adhesive. During the experiments \((n = 5–7)\), BP and heart rate (HR) were recorded every 5 min for an average of 10 s from 14.00 to 15.00, twice a week. Weekly data from each rat was averaged for analysis. Rats received an intramuscular injection of 50 mg/kg ampicillin for prophylaxis and were allowed to recover for one week following surgeries.

**In vivo gene delivery into the NTS**

Transmitter-implanted animals were re-anesthetized with isoflurane and an incision in the dorsal neck through the midline exposed the caudal dorsal medulla. Bilateral microinjections of lentiviral particles \((1 \times 10^7\) transfection units, TUs\) diluted in cerebrospinal fluid were injected into four separate sites of caudal NTS at 10 min periods using a 32-gauge Hamilton syringe \((0.5 \mu l)\): 500 μm lateral from the calamus scriptorius, 500–600 μm below the dorsal surface \((150 \text{ nl per site})\) and 350 μm lateral from the midline, 500 μm caudal to the calamus scriptorius, 500–600 μm below the dorsal surface \((100 \text{ nl per site})\).

**Baroreflex sensitivity**

Nine weeks after the lentiviral injection into the caudal NTS, rats \((n = 5–7)\) were subjected to evaluation of the baroreflex function by bolus intravenous injection of phenylephrine and sodium nitroprusside in isoflurane anesthetized condition. Reflex changes in HR induced by rapidity in BP were produced by graded doses of intravenous phenylephrine \((1 \text{ and } 3 \mu g \text{ in } 0.05 \text{ ml of saline})\) and sodium nitroprusside \((1.7 \text{ and } 5 \mu g \text{ in } 0.05 \text{ ml of saline})\); these were used to evaluate the HR baroreflex sensitivity, which was calculated by the ratio of the change in pulse interval \((\text{ms})\) to change in mean arterial pressure \((\text{MAP}, \text{mmHg})\).

**Statistical analysis**

Data are expressed as mean ± SEM. Comparisons between experimental groups were analyzed using ANOVA or Student’s \(t\) test. A value of \(p < 0.05\) is considered significant.

**Results**

Our first objective was to compare ACE2 levels in the NTS of WKY rats and SHRs in view of the established observation that the sensitivity of NTS neurons to Ang II is altered in SHR.\(^1,2\) ACE2 mRNA levels were two-fold higher in the SHR NTS compared with WKY rats (Figure 1(A)). In contrast, ACE2 protein levels and its activity were decreased by 60% and 20%, respectively, in the NTS of SHR (Figure 1 (B–D)).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** ACE2 expression in the NTS of WKY rats and SHRs. (A) ACE2 mRNA levels in the NTS: the caudal NTS were punched out bilaterally from four WKY and four SHR rats, and punches were subjected to RNA isolation protocol. Twenty-five nanograms of total RNA were utilized in real-time (RT) PCR as described in Methods. Primers and probe to 18S were used to normalize the data. Data are the mean ± SE from four rats. \(*p < 0.05\) vs. WKY rats. (B) Representative Western blot of ACE2 protein band in the NTS: 20 µg of protein isolated from the NTS punches was subjected to SDS-PAGE and Western blotting as described in Methods. (C) Quantitation of the ACE2 protein band after normalization with \(\alpha\)-tubulin. Data are the mean ± SE \((n = 8)\). \(*p < 0.05\) vs. WKY rats. (D) ACE2 enzyme activity was measured as described in Methods. Data are the mean ± SE \((n = 8)\). \(*p < 0.05\) vs. WKY rats. AFU: absolute fluorescence unit.
Next, we studied the physiological relevance of this decrease by ACE2 gene transfer in the NTS through lentiviral vector. Four weeks following lenti-GFP or lenti-ACE2 gene transfer bilaterally into the SHR NTS, rats were sacrificed and transgene expression was evaluated. Figure 2(A) shows a brainstem section of SHRs that received lenti-GFP. GFP fluorescence was remarkably restricted to the area of caudal NTS, was primarily localized in neurons, and was robust. A similar distribution of GFP fluorescence was observed in lenti-GFP or lenti-ACE2 injected WKY rats. ACE2 activity was increased 20% in lenti-ACE2 injected SHRs compared with lenti-GFP injected SHRs. These data indicate that ACE2 gene transfer increases total ACE2 activity in the NTS of the SHR.

Finally we examined HR baroreflex sensitivity, the ratio of change in pulse interval to change in MAP produced as a response to phenylephrine and sodium nitroprusside. The HR baroreflex sensitivity was compared between lenti-ACE2 treated rats and lenti-GFP treated controls. Figure 3(A) shows resting levels of MAP before a baroreflex challenging test. There were no significant changes in resting levels of MAP between lenti-GFP and lenti-ACE2 injected rats in both strains. Figure 3(B) shows representative traces of BP and HR of a lenti-GFP injected SHR (left panel) and a lenti-ACE2 injected SHR (right panel). Baroreflex bradycardia was approximately four times greater in WKY rats compared with SHRs. Lenti-ACE2 treatment had no significant effect on baroreflex bradycardia in WKY rats. However, lenti-ACE2 treatment in SHRs resulted in approximately a 60% increase in baroreflex bradycardia (0.27 ± 0.02 ms/mmHg in lenti-GFP vs. 0.44 ± 0.07 ms/mmHg in lenti-ACE2, Figure 3(C)). Baroreflex tachycardia in response to bolus injection of SNP was 0.45 ± 0.09 ms/mmHg in lenti-GFP injected WKY rats (n = 5) and 0.42 ± 0.12 ms/mmHg in lenti-ACE2 injected WKY rats (n = 5). That of lenti-GFP injected SHRs (n = 3) was 0.16 ± 0.06 ms/mmHg and lenti-ACE2 injected SHRs (n = 3) was 0.13 ± 0.02 ms/mmHg. Lenti-ACE2 treatment had no significant effect on baroreflex tachycardia in both strains. There was no significant effect on resting BP and HR up to eight weeks following lenti-ACE2 treatment in SHRs and up to four weeks in WKY rats.

**Discussion**

The major findings of our present study are that ACE2 activity is decreased in the NTS of SHR and that its gene transfer ameliorates the baroreceptor dysfunction observed in these rats. Thus, we demonstrated a direct involvement of NTS ACE2 in neural control of cardiovascular function, which supports our hypothesis that ACE2 overexpression or activation is able to overcome an intrinsic ACE2 deficiency.

Although ACE2 mRNA levels are increased in the NTS of SHR compared with the mRNA of this enzyme in the NTS of WKY rats, ACE2 protein and activity are decreased in the hypertensive strain. This observation is consistent with the report by Doobay et al. These results suggest that an altered post-transcriptional modification may exist in the NTS of hypertensive animals which leads to a reduction in the ACE2 protein synthesis. Further studies are needed to elucidate the differences in the post-transcriptional processing of ACE2 between these two strains of rats.

The ACE2 gene transfer in the NTS of hypertensive rats significantly improved the baroreflex function without affecting resting BP. Corroborating our current findings, Diz et al. reported that acute administration of MLN4760, a specific ACE2 inhibitor, into the NTS reduced the baroreflex sensitivity in normotensive Sprague-Dawley rats, thereby indicating that NTS ACE2 plays an important role in regulating baroreflex function in rats. Mechanisms involved in the improvement of baroreflex function by ACE2 gene transfer into the NTS of SHRs are not fully understood at this stage. However, as ACE2 is the main repressor of the renin-angiotensin system, the increased ACE2 activity in the NTS may directly influence the renin-angiotensin system.
Ang-(1-7)-forming enzyme from Ang II, it is tempting to speculate that overexpression of this enzyme improves the baroreflex sensitivity by shifting the balance of the ACE-Ang II-AT1 receptor axis towards the ACE2-Ang-(1-7)-Mas receptor axis of the RAS. Evidence in support of this speculation are as follows: (i) it has been well documented that antagonizing Ang II actions through AT1 receptors in the NTS leads to a remarkable improvement in the baroreflex function, 3 (ii) microinjection of Ang-(1-7) into this nucleus induces a facilitation of the baroreflex responses whereas microinjection of its antagonist attenuates the reflex3,20, and (iii) microinjections of the ACE2 inhibitor MLN4760 into the NTS produce similar effects in the baroreceptor reflex sensitivity to those observed with the use of the Ang-(1-7) receptor antagonist A-779.19,20 This suggests that the inhibition of Ang-(1-7) formation and the blockage of its actions cause impairment in the baroreflex function. Overall, these findings indicate that the ACE2-Ang-(1-7)-Mas axis is a critical modulator of the baroreflex control of HR in response to increases in arterial pressure. All components of this axis are demonstrated to be present in the NTS nucleus in support of this view.19,21

In the present study, we did not observe any effect on resting BP and HR following lenti-ACE2 transduction of the SHR NTS. This was in contrast with our previous observation that ACE2 gene transfer in the RVLM of the SHR decreased BP and HR.6 This suggests that ACE2 in these two brain areas has a different role in cardiovascular regulation. In the NTS, ACE2 regulates both the baroreceptor reflex and set point control of arterial pressure, thus playing an important role in baroreflex function. We believe that this finding is relevant since impaired baroreflex function seen in neurogenic hypertension causes increase in blood pressure variability which is a risk factor for an adverse cardiovascular outcome. Therefore, we believe that impaired baroreflex function should also be a target for neurogenic hypertension.

In summary, brain RAS is important in controlling the cardiovascular function, and its hyperactivity is linked to hypertension and cardiovascular diseases. It is imperative to control this hyperactivity if neurogenic hypertension is to be successfully treated. Our studies are relevant in this respect since they show, for the first time, that overexpression of ACE2 into the NTS improves baroreflex function on a long-term basis.

Figure 3. Effects of lenti-ACE2 administration into the NTS on baroreflex sensitivity. (A) Resting levels of MAP before baroreflex challenging test in lenti-GFP injected rats and lenti-ACE2 injected rats in both strains. (B) Representative traces of arterial pressure (AP) and heart rate (HR) in response to intravenous injection of phenylephrine (3µg) in lenti-GFP (left) and lenti-ACE2 (right) injected SHRs. Baroreflex function was examined by bolus intravenous injection of phenylephrine. (C) HR baroreflex sensitivity. HR baroreflex sensitivity was calculated by the ratio of change in pulse interval (ms) to change in mean arterial pressure (mmHg) produced by graded doses of intravenous phenylephrine (1 and 3µg in 0.05ml of normal saline). Data are the mean ± SE, n = 5–7 in each group. *p < 0.05 vs. lenti-GFP-treated SHRs.
basis in hypertensive animals. Thus, increase or activation of brain ACE2 could be a therapeutic strategy to treat neurogenic hypertension and its related pathophysiology.

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Conflict of interest

None.

References


