**Angiotensin II Inhibits Neuronal Nitric Oxide Synthase Activation Through the ERK1/2-RSK Signaling Pathway to Modulate Central Control of Blood Pressure**

Wen-Han Cheng, Pei-Jung Lu, Wen-Yu Ho, Che-Se Tung, Pei-Wen Cheng, Michael Hsiao, Ching-Jiunn Tseng

**Rationale:** Angiotensin (Ang) II exerts diverse physiological actions in both the peripheral and central nervous systems. It was reported that the activity of Ang II is higher in the nucleus tractus solitarii (NTS) of spontaneously hypertensive rats (SHRs) and that angiotensin type-1 receptors are colocalized with NAD(P)H oxidase in the neurons of the NTS, resulting in the induction of local reactive oxygen species production by Ang II. However, the signaling mechanisms of Ang II that induce hypertension remain unclear.

**Objective:** The aim of this study was to investigate the possible signaling pathways involved in Ang II–mediated blood pressure regulation in the NTS.

**Methods and Results:** Male SHRs were treated with losartan or tempol for 2 weeks, after which systolic blood pressure was observed to decrease significantly. Dihydroethidium staining showed many cells with high reactive oxygen species in the NTS of SHRs. The addition of losartan or tempol decreased the numbers of reactive oxygen species–positive cells in the NTS. The systemic administration of losartan or tempol reduced the systolic blood pressure and increased NO production. Immunoblotting and immunohistochemical analysis further showed that inhibition of Ang II activity by losartan or tempol significantly increased the expression extracellular signal-regulated kinase (ERK)1/2, ribosomal protein S6 kinase (RSK), and also increased neuronal NO synthase (nNOS) phosphorylation. RSK was also found to bind directly to nNOS and induce phosphorylation at the Ser1416 position.

**Conclusions:** Taken together, these results suggest that the ERK1/2-RSK-nNOS signaling pathway may play a significant role in Ang II–mediated central blood pressure regulation. (Circ Res. 2010;106:788-795.)

**Key Words:** angiotensin II, nucleus tractus solitarii, neuronal nitric oxide synthase, ribosomal protein S6 kinase, reactive oxygen species

The nucleus tractus solitarii (NTS), which is located in the dorsal medulla of the brain stem, is the primary site of termination of the vagus and glossopharyngeal nerves. The NTS participates in cardiovascular, gastric, and gustatory control. Our previous studies demonstrated that several neuromodulators are involved in cardiovascular control of the NTS, including ATP, adenosine, neuropeptide Y, angiotensin (Ang) II, NO, carbon monoxide, and insulin.

Ang II is a powerful vasoconstrictor in the peripheral blood system that exerts effects on the central nervous system, regulating fluid balance and the secretion of aldosterone. Hyperactivity of Ang II has been shown to play a major role in hypertension. Ang II is produced from enzymatic cleavage of angiotensinogen by renin and then by angiotensin converting enzyme. These pathological and physiological actions of Ang II are mediated through its type 1 receptor (AT1R).

Recent evidence suggests that hyperactivity of the brain renin–angiotensin system may play a critical role in mediating hypertension in spontaneously hypertensive rats (SHRs). Significant differences in AT1R density were seen in the NTS of SHRs and Wistar-Kyoto (WKY) rats, as SHRs had higher expression levels than WKY rats. The term “oxidative stress” describes chronically elevated levels of reactive oxygen species (ROS) and is associated with cardiovascular disease. ROS play a role in central autonomic networks that are involved in Ang II–mediated signaling. NAD(P)H oxidase is the main source of Ang II–induced ROS in neurons. NAD(P)H oxidase is composed of 2 catalytic subunits (gp91phox and p22phox) and 4 regulatory subunits (p47phox, p40phox, p67phox, and Rac1). Importantly, gp91phox and AT1R are colocalized in the neurons of the NTS, facilitating local ROS production by Ang II.
NO is synthesized by the enzyme NO synthase. There are 3 different types of NO synthases: neuronal NO synthase (nNOS), endothelial NO synthase (eNOS), and inducible NO synthase (iNOS).16 In vivo eNOS gene transfer experiments in the NTS caused hypotension and bradycardia.17–19 Our previous studies demonstrated that, when microinjected into the NTS, a nonselective inhibitor of NO synthase, increased arterial pressure and renal sympathetic nerve activity.5 A recent study also reported that selective inhibition of nNOS in the brain increased blood pressure in SHRs but not in WKY rats.20 Additionally, augmented expression of iNOS is associated with hypertension.21 These results indicate that the NOS system is involved in central cardiovascular regulation. Nevertheless, the relationship between elevated Ang II and reduced NO synthase activity and related modulation of cardiovascular function in the NTS has not been established.

In this study, we investigated whether inhibition of Ang II activity could induce depressor cardiovascular effects in the NTS. We determined which form of NOS could be activated by the addition of Ang II inhibitors. In addition, we investigated the downstream signaling pathway involved in Ang II effects in the NTS. Our results suggest that the extracellular signal-regulated kinase (ERK)1/2–ribosomal protein S6 kinase (RSK)–nNOS signaling pathway is involved in Ang II– and ROS-mediated modulation of blood pressure in the NTS.

![Image](http://circres.ahajournals.org/)

**Figure 1.** In situ qualitative and quantitative evaluations of ROS production in the NTS after addition of Ang II inhibitors. A, Representative red fluorescence images indicating ROS-producing cells in the NTS before and after systemic administration of losartan or tempol. The nuclei of the cells in the NTS were counterstained with DAPI and exhibited blue fluorescence. The images were photographed at ×100 and ×200 magnification. Note the reduced numbers of red fluorescent cells observed in the NTS sections of the losartan- or tempol-treated groups as compared with SHR controls. B, Bar graph representation of ROS index in the NTS of losartan- and tempol-treated and control SHR groups. The ROS index is the relative mean intensity of fluorescence of dihydroethidium. Note a significant decrease in ROS production in the NTS of the losartan- and tempol-treated group. Values are shown as means±SEM (n=6). *P<0.05 vs SHR group; #P<0.05 vs SHR group.

### Methods

#### Experimental Chemicals

All experimental drugs were purchased from Sigma-Aldrich (St Louis, Mo), except when otherwise noted.

#### Animals

Sixteen-week-old male SHRs were obtained from the National Science Council Animal Facility and housed in the animal room of Kaohsiung Veterans General Hospital (Kaohsiung, Taiwan). Humane treatment was administered at all times. The rats were kept in individual cages in a room where lighting was controlled (12 hours on/12 hours off), and the temperature was maintained between 23°C and 24°C. The rats were acclimatized to the housing conditions for 1 week and were then trained for 1 week to acclimate the animals to the procedure of indirect blood pressure measurement. The rats were randomly assigned to the 3 groups, with 6 rats per group: (1) SHR group, SHRs; (2) SHR+losartan group, SHRs fed with losartan (AT1R antagonist); (3) SHR+tempol group, SHRs fed with tempol (superoxide dismutase mimetic). The SHR+losartan group was administered losartan (30 mg/kg per day) by gavage for 2 weeks. The SHR+tempol group was given tempol (1 mmol/L), which was given in the drinking water for 2 weeks. The daily water consumption of the animals was measured. The rats were given normal rat chow (Purina, St Louis, Mo) and tap water ad libitum. All animal research protocols were approved by the Research Animal Facility Committee of Kaohsiung Veterans General Hospital.

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

#### Results

**Ang II Induces In Situ ROS Generation in the NTS**

In this study, we used SHRs as our model because higher levels of Ang II and AT1R were reported in these animals as compared with WKY rats.11,22 Previous studies suggested that in vivo Ang II increases ROS generation via NAD(P)H oxidase in NTS neurons.9 Therefore, we determined the in situ level of ROS in the NTS to establish an association between Ang II and ROS in SHRs. Figure 1A shows many red fluorescent cells observed in the NTS sections of losartan- and tempol-treated groups as compared with SHR controls. The ROS index in the NTS of losartan- and tempol-treated and control SHR groups. The ROS index is the relative mean intensity of fluorescence of dihydroethidium. Note a significant decrease in ROS production in the NTS of the losartan- and tempol-treated group. Values are shown as means±SEM (n=6). *P<0.05 vs SHR group; #P<0.05 vs SHR group.

- **Ang II** angiotensin II
- **AT1R** angiotensin II type 1 receptor
- **eNOS** endothelium NO synthase
- **ERK** extracellular signal-regulated kinase
- **iNOS** inducible NO synthase
- **MEK** mitogen-activated protein kinase–extracellular signal-regulated kinase
- **nNOS** neuronal NO synthase
- **NTS** nucleus tractus solitarii
- **P** phosphorylated
- **ROS** reactive oxygen species
- **RSK** ribosomal protein S6 kinase
- **SHR** spontaneously hypertensive rat
- **WKY** Wistar–Kyoto

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Role of Ang II in Mediating the Effect of the NTS on Blood Pressure in SHRs

To assess whether Ang II and ROS participate in the blood pressure modulatory effects of the NTS of SHRs, we investigated the inhibitory effects of losartan or tempol, on blood pressure effects in the peripheral nervous system and NTS. Figure 2A shows the time-course measurements of systolic blood pressure (SBP) from day 0 to day 28. Administration of losartan or tempol began at day 0. At day 4 after losartan or tempol treatment, the SBP decreased significantly (Figure 2A; 188±5.2 versus 155±6 and 165±5 mm Hg, P<0.05, n=6). The maximal decrease in SBP was observed 14 days after losartan or tempol treatment (184±4 versus 146±3, and 149±3 mm Hg, P<0.05, n=6). At day 15, we administration of the medication was stopped and the rats began to recover from losartan or tempol treatment. At day 28, 14 days after recovery from losartan or tempol treatment, the SBP of the treated rats had risen to the same level as observed in control rats.

Interestingly, NO production in the NTS was found to have increased significantly after treatment with losartan or tempol for 14 days (Figure 2B, blank bars; 0.15±0.05 versus 1.11±0.13 and 0.67±0.04 μmol/L per microgram of protein, P<0.05, n=6). After recovery from losartan or tempol treatment, no differences were seen in NO production between the control and experimental groups (Figure 2B, hatched bars; 0.13±0.01 versus 0.14±0.02 and 0.14±0.01 μmol/L per microgram of protein, P>0.05, n=6).

Ang II Attenuates nNOS Phosphorylation in the NTS

We further investigated whether eNOS, iNOS, or nNOS contributes to the blood pressure modulatory effects of Ang II in the NTS of SHRs. Immunoblotting analyses of proteins extracted from the NTS demonstrated that systemic treatment with losartan or tempol did not increase eNOS phosphorylation (Online Figure I, A and B; P>0.05, n=6) or iNOS protein expression (Online Figure I, C and D; P>0.05, n=6) in the NTS. Interestingly, treatment with losartan or tempol significantly increased the level of nNOS phosphorylation (Figure 2B and 3C; 1.00±0.08- versus 1.41±0.07- and 1.63±0.21-fold, P<0.05, n=6). Figure 3D and 3E shows that losartan or tempol treatment significantly increased the numbers of phosphorylated (P)-nNOS-positive cells in the NTS, as compared with SHR control rats (14±3 versus 32±5 and 35±4, P<0.05, n=6). After recovery from losartan or tempol treatment, there were no differences in nNOS phosphorylation levels between control and experimental groups (Online Figure II, A and B; P>0.05, n=6).

RSK Binds and Phosphorylates nNOS at Ser1416

To determine which kinase might be responsible for nNOS phosphorylation, we used 2 bioinformatic resources (the NetPhosK 1.0 Server [http://www.cbs.dtu.dk/services/NetPhosK] and the Kinase Phos 2.0 [http://kinasephos2.mbc.nctu.edu.tw]), to predict interrelated proteins. The results suggested that RSK could phosphorylate nNOS with a score of 0.53. We then performed coimmunoprecipitation assays to analyze whether RSK bound to nNOS. The NTS lysate was immunoprecipitated with anti-nNOS or anti-P-RSK antibody and was then probed with T-nNOS and P-RSK antibodies. The results showed P-RSK coimmunoprecipitated with nNOS (Figure 4A, lanes 2 and 3). An in vitro kinase assay was then performed to determine whether RSK directly phosphorylated nNOS in vitro. We incubated purified nNOS protein with RSK immunoprecipitated from NTS lysate, in the presence of ATP, for the reaction time indicated. Figure 4B and 4C shows the time-dependent increase in Ser1416 phosphorylation of nNOS by RSK (Figure 4B and 4C; from 0 to 2 hours; 1.00±0.08- versus 1.41±0.07- and 1.63±0.21-fold, P<0.05, n=6).

RSK Is Regulated by ERK1/2 to Modulate nNOS Activity in the NTS

RSK has previously been shown to be phosphorylated by ERK1/2 to activate the downstream signaling cascade. To determine whether RSK and ERK1/2 were activated by treatment with losartan or tempol, we performed immunoblotting analyses with antibodies specific for P-RSK and P-ERK1/2 to examine the phosphorylation levels of RSK and ERK1/2. Figure 5A and 5B shows that, after systemic treatment with losartan or tempol, the phosphorylation levels of RSK in the NTS were increased significantly in comparison with
the SHR control (1.00 ± 0.18 versus 1.57 ± 0.06, 1.44 ± 0.12-fold, \( P < 0.05, n = 6 \)). ERK1/2 phosphorylation was also significantly increased in comparison with SHR control (Figure 5A and 5B; 1.00 ± 0.33 versus 3.90 ± 0.77, and 3.42 ± 0.33-fold, for ERK1 and 1.00 ± 0.21 versus 2.51 ± 0.27, and 2.30 ± 0.29-fold, for ERK2, \( P < 0.05, n = 6 \)). After recovery from losartan or tempol treatment, there were no differences in RSK and ERK1/2 phosphorylation levels between in the control and experimental groups (Online Figure III, A and B; \( P > 0.05, n = 6 \)).

Central Administration of Losartan or Tempol Activates ERK1/2-RSK-nNOS Pathway

To explore whether central administration of losartan or tempol also upregulated ERK1/2, RSK, and nNOS activity, we performed bilateral microinjections of losartan (6 pmol/60 nL) or tempol (50 nmol/60 nL) or vehicle into the NTS of SHRs. Online Figure V, A and B, shows that, after microinjection of losartan or tempol, the phosphorylation levels of ERK1/2 and RSK in the NTS were significantly increased compared with SHR controls (1.00 ± 0.39 versus 4.57 ± 0.34- and 4.05 ± 0.53-fold for ERK1; 1.00 ± 0.59 versus 3.45 ± 0.70- and 3.59 ± 0.41-fold for ERK2; 1.00 ± 0.32 versus 3.03 ± 0.29- and 2.45 ± 0.57-fold for RSK, \( P < 0.05, n = 6 \)). nNOS\(^{S1416}\) phosphorylation was significantly increased compared with SHR controls (Online Figure V, C and D; 1.00 ± 0.21 versus 2.56 ± 0.17, and 2.41 ± 0.18-fold, for nNOS, \( P < 0.05, n = 6 \)).

Mitogen-Activated Protein Kinase–ERK Kinase Inhibitor Attenuated Losartan- or Tempol-Induced ERK1/2-RSK-nNOS Pathway Activity in the NTS

To test whether the mitogen-activated protein kinase–ERK kinase (MEK-ERK1/2)-RSK cascade might reduce losartan- or tempol-induced nNOS phosphorylation, we investigated the effect of the MEK inhibitor, PD98059 on ERK1/2, RSK and nNOS phosphorylation in the NTS. Immunoblotting analyses showed that pretreatment with PD98059 attenuated losartan- or tempol-induced ERK1/2\(^{T202/Y204}\) and RSK\(^{T359/S363}\) phosphorylation in the NTS (Online Figure VI, A and B; 1.00 ± 0.19- versus 1.22 ± 0.11- and 1.11 ± 0.07-fold for ERK1; 1.00 ± 0.11- versus 1.02 ± 0.11- and 0.99 ± 0.18-fold for ERK2; 1.00 ± 0.32- versus 0.99 ± 0.29- and 1.28 ± 0.57-fold for RSK, \( P < 0.05, n = 6 \)). nNOS\(^{S1416}\) phosphorylation levels were also reduced by PD98059 treatment (Online Figure VI, C and D; 1.00 ± 0.22 versus 1.23 ± 0.11, and 1.13 ± 0.10-fold, for nNOS, \( P > 0.05, n = 6 \)).

Discussion

In the present study, our data revealed that Ang II induces ROS production (Figure 1) to impair NO production in the NTS.
ROS in the brain are thought to contribute to the neuropathogenesis of hypertension by enhancing sympathetic nervous system activity. The essential NAD(P)H oxidase subunit gp91phox is present in somatodendritic and axonal profiles that contain AT1R in the NTS. NAD(P)H oxidase subunits are also present in neurons and astroglia in the medial NTS. In vivo, Ang II also increases ROS generation via NAD(P)H oxidase in NTS neurons. When compared with WKY rats, SHRs exhibit increased ROS production not only in the aorta, but also in the NTS. Tempol (4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl) can permeate biological membranes to scavenge superoxide anions. Tempol is not an antihypertensive drug; however, in our study, it demonstrated an antihypertensive effect. Our present study provides further support for these observations by showing the in situ expression of ROS in the NTS of SHRs. The inhibition of the AT1R by losartan and the inhibition of superoxide production by tempol decreased ROS production in the NTS of SHRs. These results also demonstrate that tempol inhibits Ang II–mediated superoxide production and hypertension, as Nishiyama et al described previously.

Our previous study showed that eNOS might play a critical role in NTS cardiovascular function via the adenosine-ERK1/2-eNOS signaling pathway. Intra-NTS eNOS gene delivery was also found to induce a depressor response in SHRs. Paton and colleagues demonstrated that eNOS-generated NO in the NTS plays a role in the control of baroreflex gain and arterial pressure. Furthermore, previous reports showed that iNOS is involved in cardiovascular modulation in the brain stem. In our present study, we did not detect reductions of eNOS or iNOS activity after inhibition of Ang II downstream of AT1R or depletion of ROS in the NTS of SHRs (Online Figure I). However, we observed increased in nNOS phosphorylation in the NTS using immunoblotting and immunohistochemistry analysis (Figure 3). This evidence suggests that nNOS might be one of the downstream targets of Ang II that is involved in NO production and that modulates blood pressure function in the NTS of SHRs. We observed only slight differences between the losartan-treated group and the tempol-treated group, ROS production, blood pressure, NO production, and nNOS phosphorylation. These results indicate that a ROS-dependent but AT1R-independent mechanism may exist. However, this mechanism remains unclear.

Interestingly, our results identified RSK as a key factor in the activation of nNOS (Figure 4B). RSK is located downstream of the Raf-MEK-ERK protein kinase cascade and contains 2 functional kinase domains: an N-terminal kinase that phosphorylates the substrates of RSK and a C-terminal kinase involved in the activation of RSK itself. A previous study demonstrated that vascular nNOS expression is selectively increased in response to platelet-derived growth factor through MEK/ERK cascade activation. In addition, epidermal growth factor stimulation was found to induce ERK1/2 activation to form RSK-nNOS complexes in the brain’s neuronal cells. RSK may phosphorylate NOS to block the accumulation of toxic
levels of NO. In our study, we verified an interaction between RSK and nNOS through a coimmunoprecipitation assay. However, our results showed RSK directly increased nNOS phosphorylation at Ser1416 in a time-dependent manner in vitro (Figure 4B and 4C). The RSK-induced phosphorylation of Ser1416, a proposed activation site for nNOS, may result in the production of NO in the NTS (Figure 2B), which in turn could modulate cardiovascular effects. Thus, we propose that the ERK1/2-RSK-nNOS pathway may participate in central blood pressure regulation by Ang II in SHRs.

We used systemic administration of losartan or tempol to block Ang II–induced ERK1/2-RSK-nNOS pathway downregulation. We corroborated these results by using central administration of losartan or tempol to block Ang II–induced ERK1/2-RSK-nNOS signaling. Because systemic inhibition of AT1R could induce nonneuronal AT1R-mediated events through multiple signaling pathways, many unknown factors might regulate blood pressure and protein expression in this context. In our experiments, microinjection of losartan or tempol into the NTS induced ERK1/2, RSK, and nNOS phosphorylation (Online Figure V), and these phosphorylation events could be blocked by central administration of PD98059 after systemic administration of losartan or tempol (Online Figure VI). Taken together, these results indicate that an ERK1/2-RSK-nNOS mechanism may exist in the NTS (Figure 6).

In our present study, SHRs were used as an animal model to investigate the molecular signaling mechanism of Ang II–induced hypertension in the NTS. High levels of Ang II in the SHRs allowed systemic administration of AT1R or ROS inhibitors and monitoring of the regulation of blood pressure induced by these inhibitors. However, we found low Ang II levels in WKY rats, a normotensive genetic model of SHRs. Previously, Mallem et al demonstrated no significant difference in SBP between losartan-treated WKY and sham control WKY rats. Sun et al also showed that losartan attenuated the increase of BP and ROS generation after administration of Ang II in WKY rats. These studies suggest that Ang II might also participate in a ROS-dependent pathway to modulate blood pressure in normotensive WKY rats. However, Qadri et al revealed that there are basic differences between the role of nNOS in SHRs and WKY rats. We also observed no significant changes in SBP or ERK1/2, RSK, or nNOS activations after systemic administration of losartan or tempol in WKY rats, as compared to control (Online Figure IV). Thus, further studies are needed to validate this proposed signaling mechanism in the NTS of WKY rats.

Intracellular ROS is increased by ligand-induced receptor activation, and accumulating evidence suggests that these endogenously derived ROS may play important roles as signaling molecules. ERK1/2 is involved in cellular differentiation, proliferation and development in neuronal cells. Wu et al demonstrated that ROS can enhance protein tyrosine kinase activity to regulate ERK1/2 phosphorylation in neuroblastoma in vitro. Chen et al also reported that activation of protein phosphatases (eg, PP2A or PP5) can inhibit hydrogen peroxide-induced ERK1/2 phosphorylation in neuronal cells. However, in our study, we showed that ROS accumulation inhibited ERK1/2 activation in the NTS and induced hypertension. ERK1/2 not only regulates neuronal development but also...
play regulatory roles in neurons in response to various physiological stimuli. The possible mechanism connecting ROS and ERK1/2 in the NTS remain to be elucidated.

In conclusion, we present a novel ERK1/2-RSK-nNOS signaling pathway in the NTS that allows us to dissect the Ang II signaling pathway regarding the inhibition of NO production in the regulation of blood pressure. Our results suggest a possible interaction between RSK and nNOS in the NTS. The present data further demonstrate that Ang II may modulate central blood pressure effects via ROS to downregulate ERK1/2, RSK and nNOS.

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Disclosures
None.

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**Novelty and Significance**

**What Is Known?**

- The renin–angiotensin system has been reported to play an important role in the development of hypertension.
- Reactive oxygen species participate in central autonomic networks that are involved in Ang II–mediated signaling.
- NO participates in blood pressure control in the NTS.

**What New Information Does This Article Contribute?**

- nNOS is a key downstream target of Ang II that is involved in NO production and regulates blood pressure in the NTS of spontaneously hypertensive rats.
- The ERK1/2–RSK–nNOS pathway participates in central blood pressure regulation by Ang II in SHRs.
- Ang II may elevate central blood pressure through reactive oxygen species production to block ERK1/2–RSK–nNOS activity.

The sympathetic nervous system has moved toward center stage in cardiovascular medicine. Recently, many studies have demonstrated that sympathetic overactivity participates in the development, maintenance, and progression of elevated blood pressure. Angiotensin (Ang) II modulates sympathetic function at many loci in the central and peripheral nervous systems and has been implicated in essential hypertension. The objective of this study was to elucidate the mechanism of Ang II–induced hypertension. By using spontaneously hypertensive rats as a model, we discover a novel extracellular signal-regulated kinase (ERK)1/2–ribosomal protein S6 kinase (RSK)–neuronal NO synthase (nNOS) signaling pathway involved in brain stem control of blood pressure by Ang II. This is also the first study to demonstrate that RSK and nNOS binding occurs in nucleus tractus solitarii (NTS) and this binding induces nNOS activation. Our findings provide new insights into central nervous system regulation of essential hypertension and may be of help in further development of therapy against this disease.
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Supplemental Material

Materials and methods

Blood pressure measurement

Using a tail-cuff method as described previously\(^1\) (Model MK-2000 Storage Pressure Meter, Muromachi Kikai, Tokyo, Japan), the systolic blood pressure (SBP) and heart rate were measured prior to the start of losartan and tempol treatments (day 0). The animals were placed in the fixer for 30 mins. In this method, the reappearance of pulsation on a digital display of the blood pressure cuff was detected by a pressure transducer and amplified and recorded as the SBP. During the measurement, ten individual readings were obtained in rapid sequence. The highest and the lowest readings were dropped from consideration, and the remaining eight readings were averaged.

**In situ detection of ROS in NTS**

The endogenous in vivo ROS production of the NTS was determined by dihydroethidium (DHE) (Invitrogen, Carlsbad, CA, USA) staining.\(^2\) The NTS, dissected from studied rats, were quickly frozen, embedded in OCT, and then placed in liquid nitrogen. Cryostat slices (30 μm) were stained in the dark for 30 mins at 37 °C with a 1 μM solution of DHE. The samples were analyzed using fluorescence microscopy and the Zeiss LSM Image (Carl Zeiss MicroImaging, Göttingen, Germany) program.

Measurement of NO in the NTS

Samples were deproteinized using Microcon YM-30 centrifugal filter units (Millipore, Bedford, MA, USA). The amount of total NO in the samples was determined by a modification in the procedure based on the purge system of Sievers Nitric Oxide Analyzer (NOA 280i) (Sievers Instruments, Boulder, CO, USA), which involves the use of chemiluminescence.\(^3\) Samples (10 μl) were injected into a reflux column containing 0.1 mol/L VCl\(_3\) in 1 mol/L HCl at 90 °C to reduce any nitrates and nitrites (NOx) into NO. NO then combined with the O\(_3\) produced by the analyzer to form NO\(_2\). The resulting emission from the excited NO\(_2\) was detected by a photomultiplier tube and recorded digitally (mV). The values were then interpolated to a standard curve of NaNO\(_3\) concentrations determined concurrently. The measurements were made in triplicate for each sample. The measured NO levels were corrected for the NTS of the studied rats.

**Intra-NTS Microinjection**

The preparation of animals for intra-NTS microinjection and the methods used to locate the NTS have been described previously.\(^4\) Briefly, a polyethylene cannula was inserted into the femoral vein for fluid supplementation, and BP was measured via a femoral-artery cannula by pressure transducer and polygraph (Gould, Cleveland, OH, USA). HR was monitored by a tachograph preamplifier (Gould). To verify that the needle tip of the glass electrode was exactly in the NTS, L-glutamate (0.154 nmol/60 nL) was microinjected. This would induce a characteristically abrupt decrease in BP
(ΔBP ≥ 35 mm Hg) and HR (ΔHR ≥ 50 bpm) if the needle tip was located precisely in the NTS.

Three groups of SHR (6 rats per group) received microinjection of losartan (2 nmol/60 nL), tempol (50 nmol/60 nL) or vehicle in the unilateral NTS, and changes in BP and HR were measured.

After the SHR were given losartan or tempol 14 days, a MEK inhibitor, PD98059 (6 pmol/60 nL), was microinjected into the NTS. Vehicle was microinjected into the NTS of SHR control rats.

Immunoblotting analysis

Groups of rats (6 rats per group) were enrolled in the experiment. The NTS was dissected by micropunch (1-mm inner diameter) from a 1-mm thick brainstem slice at the level of the obex under a microscope. Total protein was prepared by homogenizing the NTS tissue in lysis buffer with a protease inhibitor cocktail and a phosphatase inhibitor cocktail, and then incubated for 1 hour at 4 °C. Protein extracts (20 μg/sample assessed by BCA protein assay, Pierce Chemical Co., Rockford, IL, USA) were resolved on a 6% polyacrylamide gel and transferred to a PVDF membrane (GE Healthcare, Buckinghamshire, UK). The membranes were incubated with the appropriate anti-P-RSK T359/S363, anti-P-ERK1/2 T202/Y204, anti-RSK, anti-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA), anti-P-nNOS S1416 (Abcam, Cambridge, UK), anti-nNOS (Millipore, Billerica, MA, USA), anti-P-eNOS S1177, anti-eNOS (BD Biosciences, San Jose, CA, USA) and anti-iNOS (Transduction Laboratories, Lexington, KY, USA) antibodies. These antibodies were diluted 1:1000 in PBST containing bovine serum albumin and were incubated with membranes overnight at 4 °C. Membranes were then incubated with an HRP-labeled goat anti-rabbit secondary antibody at 1:10000. The membranes were developed with the ECL-Plus detection kit (GE Healthcare).

Immunohistochemistry analysis

After perfusion with 0.9% normal saline, the rat brainstems were fixed immediately in 4% formaldehyde overnight and embedded in paraffin. The brainstems were sectioned coronally at 5 μm thickness. The sections were deparaffinized, quenched in 3% H2O2/methanol, microwaved in citric buffer (10 mM, pH 6.0), blocked in 5% goat serum, and incubated with an anti-P-nNOS S1416 antibody overnight at 4 °C. Next, sections were incubated with biotinylated secondary antibodies (1:200; Vector Laboratories, Burlingame, CA, USA) for 1 hour and in AB complex (1:100) for 30 minutes at room temperature. The sections were visualized with a DAB substrate kit (Vector Laboratories) and counterstained with hematoxylin. The sections were then photographed with a microscope mounted with a CCD camera.

Co-immunoprecipitation assay and in vitro kinase assay

The NTS was dissected by micropunch (1-mm inner diameter) from a 1-mm thick brainstem slice at the level of the obex under a microscope. Total protein was prepared by homogenizing the NTS tissue in lysis buffer with a protease inhibitor cocktail and a phosphatase inhibitor cocktail and was incubated for 1 hour at 4 °C. The resulting supernatant was incubated with 5 μl of a rabbit
anti-nNOS or anti-P-RSK\textsuperscript{T359/S363} antibodies. The Catch and Release immunoprecipitation system (Millipore) was used, according to the manufacturer's instructions. The proteins were eluted in 70 μl of elution buffer and subjected to immunoblotting analysis using anti-nNOS and anti-P-RSK\textsuperscript{T359/S363} antibodies.

For the \textit{in vitro} kinase assay, a co-immunoprecipitation experiment using anti-RSK and anti-nNOS antibodies was performed and the RSK-nNOS complex was eluted by the Catch and Release immunoprecipitation system. The kinase reaction was initiated by the addition of kinase buffer (100 mM HEPES, pH 7.4, 1 mM DTT, 10% Glycerol, 100 mM ATP, 1M MgCl\textsubscript{2}), and the phosphorylation reactions were terminated with the addition of 2X sample buffer and boiled for 10 minutes. The phosphorylation of nNOS was determined by immunoblotting analysis using anti-P-nNOS\textsuperscript{S1416} and anti-nNOS antibodies.

\textbf{Statistical analysis}

A paired Student's \textit{t}-test for comparing blood pressure measurements and counting P-nNOS\textsuperscript{S1416}-expressing cells, and a one-way analysis of variance (ANOVA) with Scheffe post-hoc, comparison were applied to compare group differences. Differences with P<0.05 were considered significant. All data are expressed as means ± SEM.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.
Online Figure 1. Quantitative immunoblotting analysis of eNOS phosphorylation and iNOS expression in the NTS following treatment with losartan or tempol. A. Immunoblots depicting the levels of P-eNOS\textsuperscript{S1177} protein in the NTS of SHR (lane 1), losartan-treated SHR (lane 2), and tempol-treated SHR (lane 3). B. Densitometric analysis of P-eNOS\textsuperscript{S1177} protein levels before and after treatment with losartan or tempol. Note that there are no significant differences in eNOS\textsuperscript{S1177} phosphorylation levels in the NTS between SHR controls and the losartan- or tempol-treated groups. Bar values are shown as means ± SEM, n=6. C. Immunoblot showing iNOS protein levels in the NTS of SHR (lane 1), losartan-treated SHR (lane 2), and tempol-treated SHR (lane 3). D. Densitometric analysis of iNOS protein levels before and after treatment with losartan or tempol. Note that there are no significant difference in iNOS protein levels in the NTS between SHR controls and the losartan- or tempol-treated groups. Bar values are shown as means ± SEM, n=6.
**Online Figure II.** Quantitative immunoblotting and immunohistochemistry analysis of nNOS\textsuperscript{S1416} phosphorylation in the NTS after recovery from losartan or tempol treatment. **A.** Immunoblot showing low P-nNOS\textsuperscript{S1416} protein levels in the NTS of SHR after recovery from losartan or tempol treatment. **B.** Densitometric analysis of P-nNOS\textsuperscript{S1416} levels before and after treatment with losartan or tempol. Bar values are shown as means ± SEM, n=6. \(*P<0.05\) vs. lane 1 and \(#P<0.05\) vs. lane 1. Notably, there were no significant differences in nNOS phosphorylation levels in the NTS between SHR controls and the losartan or tempol groups.
Online Figure III. Quantitative immunoblotting analysis of RSK and ERK1/2 phosphorylation in the NTS after recovery from losartan or tempol treatment. A. Immunoblot showing P-RSK\(^{T359/S363}\) and P-ERK1/2\(^{T202/Y204}\) protein levels in NTS of SHR after recovery from losartan or tempol treatment. There were no significant differences in RSK or ERK1/2 phosphorylation levels in the NTS between SHR controls and the losartan or tempol treated groups. B. Densitometric analysis of P-RSK\(^{T359/S363}\) and P-ERK1/2\(^{T202/Y204}\) protein levels after recovery from losartan or tempol. Bar values are shown as means ± SEM, n=6.
Online Figure IV. Blood pressure and protein levels in the NTS of WKY rats after treatment with losartan or tempol. A. Time-course tracing of SBP in WKY after systemic administration of losartan or tempol for 14 days. Filled circles 「●」 represent WKY rats, open circles 「 ○ 」represent WKY + losartan, and inverted filled triangles 「▼」represent WKY + tempol. SBP was measured on days 0, 4, 7, 11, 14. Note that no significant differences in SBP were observed at day 14 between WKY controls and losartan- or tempol-treated WKY rats. B. Immunoblot showing P-RSK$^{T359/S363}$ and P-ERK1/2$^{T202/Y204}$ protein levels in NTS of WKY after losartan or tempol treatment. There were no significant differences in RSK or ERK1/2 phosphorylation levels in the NTS of WKY controls and the losartan- or tempol-treated groups. C. Densitometric analysis of P-RSK$^{T359/S363}$ and P-ERK1/2$^{T202/Y204}$ protein levels after losartan or tempol treatment. D. Immunoblot showing no significant differences in P-nNOS$^{S1416}$ protein levels in the NTS of WKY controls and the losartan- or tempol-treated groups. E. Densitometric analysis of P-nNOS$^{S1416}$ protein levels after losartan or tempol treatment. Bar values are shown as means ± SEM, n=6.
Online Figure V. Quantitative immunoblotting analysis of ERK1/2, RSK and nNOS phosphorylation in the NTS following microinjection of losartan or tempol. A. Immunoblot depicting low levels of P-RSK\textsuperscript{T359/S363} and P-ERK1/2\textsuperscript{T202/Y204} proteins in the NTS of SHR. Microinjection of losartan or tempol increased the levels of P-RSK\textsuperscript{T359/S363} and P-ERK1/2\textsuperscript{T202/Y204} proteins. B. Densitometric analysis of P-eNOS\textsuperscript{S1177} protein levels with or without losartan or tempol treatment. C. Immunoblot depicting low levels of P-nNOS\textsuperscript{S1416} protein in the NTS of SHR. Losartan or tempol treatment increased the level of P-nNOS\textsuperscript{S1416} protein. D. Densitometric analysis of P-nNOS\textsuperscript{S1416} protein levels with or without losartan or tempol. Bar values are shown as means ± SEM, n=6. *P<0.05 vs. SHR and #P<0.05 vs. SHR.
Online Figure VI. Quantitative immunoblotting analysis of RSK, ERK1/2 and nNOS phosphorylation in the NTS of SHR after losartan or tempol treatment with PD98059. A. Immunoblot showing P-RSK T359/S363 and P-ERK1/2 T202/Y204 protein levels in the NTS of SHR after losartan or tempol treatment with PD98059. There were no significant differences in RSK or ERK1/2 phosphorylation levels in the NTS of SHR controls and the losartan- or tempol-treated groups after PD98059 administration. B. Densitometric analysis of P-ERK1/2 T202/Y204 and P-RSK T359/S363 protein levels after losartan or tempol treatment with PD98059. C. Immunoblot also showing no significant differences P-nNOS S1416 protein levels in the NTS of SHR controls and the losartan- or tempol-treated groups after PD98059 administration. D. Densitometric analysis of P-nNOS S1416 protein levels after losartan or tempol treatment with PD98059. Bar values are shown as means ± SEM, n=6.

