Abstract—Enhancement of the cardiac sympathetic afferent reflex (CSAR) contributes to sympathetic excitation in hypertension. The aim of the present study was to determine whether angiotensin (Ang)-(1–7) in the rostral ventrolateral medulla (RVLM) modulated the enhanced CSAR and sympathetic activation, and the signaling pathways that mediated these effects in the 2-kidney, 1-clip renovascular hypertension model. Cardiac sympathetic afferent reflex was evaluated using renal sympathetic nerve activity and mean arterial pressure responses to epicardial capsaicin application in anesthetized sinoaortic-denervated and cervical-vagotomized rats. RVLM microinjection of Ang-(1–7) induced greater increases in renal sympathetic nerve activity and mean arterial pressure, and greater enhancement in CSAR in 2-kidney, 1-clip rats than in sham-operated rats, which was blocked by Mas receptor antagonist A-779, adenyl cyclase inhibitors SQ22536 and MDL-12,330A, and protein kinase A inhibitors rp-adenosine-3′,5′-cyclic monophosphorothionate and H-89. Mas receptor expression in RVLM was increased in 2-kidney, 1-clip rats. Treatment with A-779, SQ22536, MDL-12,330A, rp-adenosine-3′,5′-cyclic monophosphorothionate, or H-89 in RVLM inhibited CSAR and decreased renal sympathetic nerve activity and mean arterial pressure in 2-kidney, 1-clip rats, whereas cAMP analogue dibutyryl-cAMP had the opposite effects. Ang-(1–7) in RVLM increased, whereas A-779 decreased the cAMP level and the epicardial capsaicin application-induced increases in the cAMP level in RVLM. These results indicate that Ang-(1–7) in the RVLM enhances the CSAR and increases the sympathetic outflow and blood pressure via Mas receptor activation. The increased endogenous Ang-(1–7) and Mas receptor activity in RVLM contributes to the enhanced CSAR and sympathetic activation in renovascular hypertension, and the cAMP-protein kinase A pathway is involved in these Ang-(1–7)–mediated effects in the RVLM. (Hypertension. 2013;61:820–827.) • Online Data Supplement

Key Words: angiotensin-(1–7) □ cAMP-PKA pathway □ hypertension □ rostral ventrolateral medulla □ sympathetic activity

Numerous studies have shown that sympathetic activity is enhanced in patients with essential or secondary hypertension and in various hypertensive models and that excessive sympathetic output contributes to hypertension pathogenesis and progression of organ damage. Therapeutic targeting of sympathetic activation is considered to be an antihypertensive strategy. The cardiac sympathetic afferent reflex (CSAR) is a sympathoexcitatory cardiovascular positive-feedback reflex. Our previous studies demonstrate that the CSAR is enhanced in renovascular hypertension and contributes to sympathetic activation and hypertension.

The rostral ventrolateral medulla (RVLM) is the vasomotor center that controls basal sympathetic nerve activity and arterial pressure. The paraventricular nucleus is an important component of the central neurocircuitry of the CSAR. Our previous study has shown that RVLM superoxide anions are necessary for the CSAR, as well as for the enhanced effect of angiotensin (Ang) II on the CSAR in the paraventricular nucleus, suggesting an important role for the RVLM in CSAR control. Ang-(1–7) is an important biologically active peptide in the renin–Ang system family and is involved in regulating sympathetic outflow and cardiovascular activity. Ang-(1–7) is either formed directly from Ang II or indirectly from Ang I by Ang-converting enzyme 2. Most of the effects of Ang-(1–7) are mediated by the Mas receptor and are selectively blocked by its specific antagonist d-Alanine-Ang-(1–7) (A-779). There are abundant Mas receptors in the brain and, more importantly, in cardiovascular-related areas, including...
the RVLM, nucleus of the solitary tract, parvocellular and magnocellular portions of the paraventricular nucleus, and other areas. Furthermore, Mas staining is predominant in the neuron membrane.23 RVLM Ang-(1–7) microinjection elicits a pressor response in rats.24,25,38 Our recent study has shown that Ang-(1–7) in the RVLM enhances the CSAR and increases renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP), whereas A-779 microinjection into the RVLM decreases the RSNA and MAP and inhibits the CSAR in normal rats.32 However, it is not known whether Ang-(1–7) in the RVLM is involved in the enhanced CSAR and excessive sympathetic activation in hypertension.

A recent study demonstrates that Mas receptor activation by Ang-(1–7) increases the intracellular cAMP level and activates protein kinase A (PKA) and that inhibition of either adenyl cyclase (AC) or PKA activity attenuates Ang-(1–7)–induced extracellular signal-regulated kinase 1/2 activation in glomerular mesangial cells.33 Ang-(1–7) inhibits vascular growth via prostacyclin-mediated cAMP production and PKA activation.34 These results demonstrate that the AC-PKA signaling pathway regulates Ang-(1–7) function in some peripheral tissues. However, whether the AC-PKA pathway is involved in Ang-(1–7)–mediated effects in the RVLM in hypertension is not well understood. The present study was designed to determine whether Ang-(1–7) in the RVLM contributed to the enhanced CSAR and sympathetic activation that occurred in renovascular hypertension and whether the AC-PKA pathway was involved in the Ang-(1–7)–mediated effects on the CSAR and sympathetic outflow in renovascular hypertensive rats.

**Materials and Methods**

All of the experiments were performed in male Sprague–Dawley rats. The procedures were approved by the Nanjing Medical University Experimental Animal Care and Use Committee and compiled with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85-23, revised 1996). The Goldblatt 2-kidney, 1-clip (2K1C) method was used to elicit renovascular hypertension as in our previous reports.16–18

Acute experiments were performed at the end of the fourth week after the clipping or sham surgery. RSNA and MAP were continuously recorded in sinoaortic-denervated and cervical-vagotomized rats under anesthesia with intraperitoneal administration of urethane (800 mg/kg) and α-chloralose (40 mg/kg), as reported previously.35,36 CSAR was evaluated using RSNA and MAP responses to epicardial capsaicin (1.0 nmol) application. The rats were placed in a stereotaxic frame, and the bilateral RVLM microinjections were performed. The Mass receptor expression in the RVLM was determined with Western blotting and immunohistochemistry. Ang-(1–7) and cAMP levels in the RVLM were determined with ELISA. Rat echocardiography was performed with an ultrasound system. An expanded Methods section is available in the online-only Data Supplement.

Ang-(1–7) and α-Alanine-Ang-(1–7) (A-779) were purchased from Bachem (Bubendorf, Switzerland); rt-adenosine-3',5'-cyclic monophosphorothioate (Rp-cAMP), N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide dimethylether (H-89), dibutyl-cAMP (db-cAMP), 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536), cAMP-sensory lymphocytopenyl)-azacyclotridec-1-en-2-amine and LS-22536, cAMP-PKA pathway was involved in the Ang-(1–7)–mediated enhancement of the CSAR in the 2K1C rats was much greater than in the sham rats. The negative effects of Ang-(1–7) in the 2K1C rats were greater than in the sham rats (Figure 1B). Ang-(1–7)–mediated enhancement of the CSAR in the 2K1C rats was much greater than in the sham rats (Figure 1B). Representative recordings of the effects of RVLM microinjection of saline and the high doses of Ang-(1–7) on the CSAR in sham and 2K1C rats were shown in the online-only Data Supplement (Figure S2). RVLM Ang-(1–7) microinjection did not have a significant effect on echocardiographic data in both 2K1C and sham rats (Table S2).

**Echocardiography**

Echocardiography was used to evaluate left ventricular function and geometry changes. Compared with the sham rats, 4 weeks after clipping, the interventricular septal thickness in diastole, interventricular septal thickness in systole, left ventricular posterior wall thickness in diastole, left ventricular posterior wall thickness in systole, left ventricular mass and left ventricular mass/body weight were increased, whereas left ventricular end-diastolic diameter, left ventricular end-systolic diameter, fractional shortening, and ejection fraction did not significantly change in 2K1C rats (Table S2).

**Effects of Ang-(1–7)**

RVLM microinjection of Ang-(1–7) increased the baseline RSNA and MAP in both 2K1C and sham rats in a dose-dependent manner. Ang-(1–7) treatment in the 2K1C rats caused larger increases in the RSNA and MAP than in the sham rats (Figure 1A). Both the middle and high doses of Ang-(1–7) significantly augmented the CSAR in sham and 2K1C rats, although the CSAR was already enhanced in 2K1C rats. The Ang-(1–7)–mediated enhancement of the CSAR in the 2K1C rats was much greater than in the sham rats (Figure 1B). Representative recordings of the effects of RVLM microinjection of saline and the high doses of Ang-(1–7) on the CSAR in sham and 2K1C rats were shown in the online-only Data Supplement (Figure S2). RVLM Ang-(1–7) microinjection did not have a significant effect on echocardiographic data in both 2K1C and sham rats (Table S2).

**Effects of A-779**

Microinjecting 3 doses of Mas receptor antagonist A-779 into the RVLM dose-related decreased the baseline RSNA and MAP and inhibited CSAR in both 2K1C and sham rats. Compared with saline, only the high dose of A-779 significantly inhibited the RSNA, MAP, and CSAR in sham rats, whereas both the middle and high doses of A-779 inhibited these values in the 2K1C rats. The negative effects of A-779 in the 2K1C rats were greater than in the sham rats (Figure 2). A-779 pretreatment in the RVLM blocked the effects of Ang-(1–7) on the RSNA, MAP, and CSAR in both sham and 2K1C rats but had no effect on Ang II–induced RSNA, MAP, and CSAR responses in the RVLM (Figure 3). RVLM microinjection of A-779 had no significant effect on echocardiographic data in both 2K1C and sham rats (Table S2).
Ang-(1–7) Level and Mas Receptor Expression
There was no significant difference in the Ang-(1–7) level in the RVLM between sham and 2K1C rats (Figure 4A), but Mas receptor protein expressions determined by Western blotting (Figure 4B) and immunoreactivity (Figure 4C) in RVLM were significantly increased in 2K1C rats compared with sham rats. Representative photos showing the Mas receptor immunohistochemistry in the RVLM in sham and 2K1C rats were shown in the online-only Data Supplement (Figure S3).

Figure 1. Effects of rostral ventrolateral medulla (RVLM) microinjection of saline and 3 doses of angiotensin (Ang)- (1–7) (0.03, 0.30, and 3.00 nmol) on the baseline renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP; A) and the cardiac sympathetic afferent reflex (CSAR; B) in sham-operated (sham) and 2-kidney, one-clip (2K1C) rats. The CSAR was evaluated by the RSNA and MAP response to epicardial capsaicin (1 nmol) application. Values are means±SE. *P<0.05 vs saline. †P<0.05 vs sham. n=6 for each group.

Effects of SQ22536, MDL-12,330A, db-cAMP, Rp-cAMP, and H-89
RVLM microinjection of AC inhibitor SQ22536 or MDL-12,330A decreased RSNA and MAP in 2K1C rats and attenuated CSAR in both sham and 2K1C rats. db-cAMP, a cAMP analogue, elicited greater increases in RSNA and MAP and greater enhancement of CSAR in 2K1C rats than in sham rats. PKA inhibitors Rp-cAMP and H-89 in RVLM produced similar results as the AC inhibitors (Figure 5). RVLM pretreatment

Figure 2. Effects of saline and 3 doses of α-Alanine-Ang-(1–7) (A-779) (0.03, 0.30, and 3.00 nmol) microinjection into the rostral ventrolateral medulla (RVLM) on the baseline renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP; A) and the cardiac sympathetic afferent reflex (CSAR; B) in sham-operated (sham) and 2-kidney, one-clip (2K1C) rats. The CSAR was evaluated by the RSNA and MAP responses to epicardial capsaicin (1 nmol) application. Values are means±SE. *P<0.05 vs saline. †P<0.05 vs sham. n=6 for each group.
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with SQ22536, MDL-12,330A, Rp-cAMP, and H-89 abolished Ang-(1–7)–mediated RSNA, MAP, and CSAR enhancement in both sham and 2K1C rats, but db-cAMP pretreatment failed to augment the effects of Ang-(1–7) (Figure 6).

cAMP Level

The RVLM microinjection of Ang-(1–7) caused greater increases in cAMP level in 2K1C rats compared with the sham rats, and A-779 treatment decreased cAMP level in 2K1C rats (Figure 7A). Epicardial capsaicin application increased the cAMP level in RVLM in both sham and 2K1C rats, but this effect was greater in 2K1C rats (Figure 7B). RVLM pretreatment with A-779 inhibited, whereas Ang-(1–7) enhanced, the increase in cAMP level after epicardial capsaicin application (Figure 7C).

Discussion

Previous studies from our laboratory demonstrate that the CSAR is enhanced in renovascular hypertensive rats and spontaneously hypertensive rats and contributes to sympathetic activation and hypertension. The present study demonstrates new findings that Mas receptor activation with Ang-(1–7) in the RVLM increases sympathetic outflow and blood pressure and enhances the CSAR. Endogenous Ang-(1–7) and Mas receptor activity contributes to the enhanced CSAR and excessive sympathetic activation in renovascular hypertensive rats. A cAMP-PKA pathway is involved in the Ang-(1–7)–mediated effects in the RVLM.

The discovery of Ang-(1–7) in the last decade adds a new component to the renin–Ang system family. Central Ang-(1–7) is involved in regulation of sympathetic outflow and cardiovascular activity. Dense Mas receptor immunoreactivity is found in the RVLM. Ang-(1–7) microinjection into the RVLM elicits a pressor response in rats. Mas receptor antagonist A-779 in RVLM induces a pressor response in rats harboring the mouse renin Ren-2 gene, TGR(mREN2)27. Our recent study has shown that Ang-(1–7) microinjection into the RVLM enhances CSAR and increases RSNA and MAP, whereas A-779 in the RVLM inhibits the CSAR and decreases RSNA and MAP in normal rats. In the present study, microinjection of 3 doses of Ang-(1–7) into the RVLM increased the baseline RSNA and MAP in both 2K1C and sham rats in a dose-dependent manner, and the 2K1C rats were more sensitive to Ang-(1–7). The CSAR was enhanced in 2K1C rats, which was consistent with our previous findings. The middle and high doses of Ang-(1–7) augmented CSAR in both 2K1C and sham rats, and the Ang-(1–7)–mediated effect on the CSAR in 2K1C rats was much greater than in the sham rats. The specific Mas receptor antagonist A-779 blocked Ang-(1–7)–mediated effects, including increasing the RSNA and MAP and enhancing the CSAR in both 2K1C and sham rats. These results suggest that exogenous Ang-(1–7) in the RVLM increases RSNA and MAP and enhances the CSAR, which is mediated by Mas receptors, and Ang-(1–7) was more reactive in 2K1C rats compared with sham rats. We found that Mas receptor expression in the RVLM determined by either Western blotting or immunohistochemistry was increased in 2K1C rats compared with sham rats, although no significant difference in the Ang-(1–7) level in RVLM was observed between sham and 2K1C rats, which suggests that Ang-(1–7) and Mas receptor activity in the RVLM is enhanced in renovascular hypertension. Therefore, a potential mechanism for the intensified responses to Ang-(1–7) in 2K1C rats may be induction of increased Mas receptor expression in the RVLM.

A-779 treatment reportedly blocks the antidiuretic effect of Ang-(1–7) but does not affect the antidiuretic effects of vasopressin, the dipsogenic pressor effects of Ang II, or the contractile effects of Ang III, bradykinin, or substance P on the rat ileum. In the RVLM, the Ang-(1–7) microinjection-induced pressor effect is completely blocked by A-779 but is not blocked by Ang II type 1 or type 2 receptor antagonists; conversely, the
Ang II–induced pressor effect is not affected by A-779. A-779 does not compete for 125I-Ang II binding to adrenocortical membranes and adrenomedullary membranes.28 At medullary sites, specific and high-intensity binding for rhodamine-Ang-(1–7) is completely displaced by Mas antibody or by A-779. These results demonstrate that A-779 is a potent, specific, and selective Ang-(1–7) antagonist.29 In the present study, RVLM pretreatment with A-779 had no effect on Ang II–induced increases in the RSNA, MAP, and CSAR, which further verified the specificity of A-779 as a Mas receptor antagonist.

RVLM microinjection of 3 doses of Mas receptor antagonist A-779 dose-dependently decreased the baseline RSNA and MAP and inhibited CSAR to a level that was lower than normal in both 2K1C and sham rats. The negative effects of A-779 in 2K1C rats were much greater than those in sham rats. These results, combined with the increasing Mas receptor expression data in RVLM in 2K1C rats, indicate that the increasing endogenous Ang-(1–7) and Mas receptor activity contributes to the enhanced CSAR, excessive sympathetic outflow, and high blood pressure in renovascular hypertension. A-779 in RVLM also decreased the RSNA, MAP, and CSAR in sham rats, suggesting that endogenous Ang-(1–7) in the RVLM is involved in the tonic control of the CSAR, sympathetic activity, and blood pressure in physiological status.

The effects of Ang-(1–7) and A-779 in RVLM on left ventricular function were also evaluated. Four weeks after clipping, echocardiography revealed a significant increase in the left ventricular wall thickness (interventricular septum and posterior wall) and an increase in the left ventricular mass/body weight in the 2K1C rats compared with sham rats, which suggests that 2K1C rats developed cardiac hypertrophy. There were no significant differences in left ventricular end-diastolic diameter, left ventricular end-systolic diameter, fractional shortening, and ejection fraction between 2K1C and sham rats, which suggests that left ventricular function in 2K1C rats does not change 4 weeks after clipping. However, there were no significant echocardiographic changes in either 2K1C or sham rats 8 minutes after RVLM Ang-(1–7) or A-779 microinjection, which indicates that Ang-(1–7) or A-779 in RVLM does not influence the left ventricular function in a short time.

It has been reported that Ang-(1–7)-induced extracellular signal-regulated kinase 1/2 activation in glomerular mesangial cells is cAMP/PKA dependent.33 Ang-(1–7) inhibits vascular growth through prostacyclin-mediated production of cAMP and activation of PKA. The PKA inhibitor blocks, whereas cAMP mimics, the Ang-(1–7) effect on Ang II–stimulated proximal tubule Na⁺-ATPase.41 However, whether the cAMP-PKA pathway is involved in Ang-(1–7)–mediated CSAR enhancement and sympathetic activation in the RVLM in renovascular hypertension is unknown. SQ22536 is a specific membrane-permeable AC inhibitor that completely blocks the rise in cAMP evoked by AC activation,42 and Rp-cAMP is a specific membrane-permeable PKA inhibitor.34,43 db-cAMP is a cAMP analogue, and exposure of Down syndrome human fetal skin fibroblasts to db-cAMP stimulates PKA activity.44 In the present study, both RVLM SQ22536 and Rp-cAMP pretreatment abolished Ang-(1–7)-induced RSNA, MAP, and CSAR elevation in both 2K1C and sham rats. To control for nonspecific effects of SQ22536 or Rp-cAMP, the effects of MDL-12,330A, a second AC inhibitor,45 and H-89, a second PKA inhibitor,43,44 were also determined. We found that MDL-12,330A or H-89 treatment produced similar effects as SQ22536 or Rp-cAMP. Furthermore, RVLM Ang-(1–7) microinjection caused a greater increase in cAMP level in RVLM in 2K1C rats compared with the sham rats, whereas A-779 in RVLM decreased the cAMP level in 2K1C rats. These results indicate that the cAMP-PKA pathway in the RVLM mediates the effects of Ang-(1–7) on the RSNA, MAP, and CSAR in renovascular hypertension.

The addition of SQ22536 or Rp-cAMP alone in the RVLM decreased but the cAMP analogue db-cAMP increased the baseline RSNA and MAP in 2K1C rats, suggesting that the cAMP-PKA pathway is involved in sympathetic activation and high blood pressure in renovascular hypertension. We also found that SQ22536 or Rp-cAMP alone significantly

![Figure 4](https://example.com/fig4.png)

**Figure 4.** Angiotensin (Ang)-(1–7) level (A), Mas receptor protein expression determined by Western blotting (B) and Mas receptor immunohistochemistry (C) in the rostral ventrolateral medulla (RVLM) in sham-operated (sham) and 2-kidney, one-clip (2K1C) rats. Values are mean±SE. *P<0.05 vs sham rats. n=5 for each group.
attenuated CSAR not only in 2K1C rats but also in sham rats, whereas db-cAMP treatment enhanced the CSAR more in 2K1C rats than in sham rats. The second AC inhibitor MDL-12,330A or the second PKA inhibitor H-89 had effects similar to SQ22536 or Rp-cAMP. Epicardial capsaicin application to induce CSAR increased the cAMP level in RVLM more in the 2K1C rats than in the sham rats, and this effect was inhibited by RVLM A-779 pretreatment and augmented by Ang-(1–7) pretreatment. These results suggest that the cAMP-PKA pathway contributes to tonic control of the CSAR and that endogenous Ang-(1–7) and Mas receptors in the RVLM are involved in the tonic control of the CSAR by activating the cAMP-PKA pathway.

Conclusions
Ang-(1–7) in the RVLM enhances the CSAR and increases sympathetic output and blood pressure by activating Mas receptors. The increased endogenous Ang-(1–7) and Mas receptor activity in the RVLM contributes to the enhanced CSAR and sympathetic activation in renovascular hypertension through a mechanism that is mediated by the cAMP-PKA pathway.
Perspectives

Excessive sympathetic activation contributes to hypertension pathogenesis and progression of organ damage. We have demonstrated that the CSAR is enhanced in renovascular hypertension and contributes to sympathetic activation and hypertension. The present study demonstrates several points for the first time: (1) Ang-(1–7) in the RVLM enhances CSAR and increases sympathetic output and blood pressure by Mas receptor activation; (2) increased endogenous Ang-(1–7) and Mas receptor activity contributes to the enhanced CSAR and sympathetic activation in renovascular hypertension; (3) and the cAMP-PKA pathway is involved in Ang-(1–7)–mediated effects in the RVLM. Although further studies are needed to address the clinical relevance of these findings, the present results support the notion that, in the RVLM, Ang-(1–7) and the cAMP-PKA pathway may offer promising new therapeutic targets to counteract the enhanced CSAR and sympathetic activation that is found in renovascular hypertension.

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Disclosures

None.

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Novelty and Significance

What Is New?

- Ang-(1–7) in the RVL enhances the CSAR and increases sympathetic output and blood pressure through Mas receptor activation.
- Increased endogenous Ang-(1–7) and Mas receptor activity contributes to the enhanced CSAR and sympathetic activation in renovascular hypertension.
- The cAMP-PKA pathway is involved in the Ang-(1–7)–mediated effects in rats.

- Ang-(1–7) and the cAMP-PKA pathway in the RVL may offer promising new therapeutic targets to counteract the enhanced CSAR and sympathetic activation in renovascular hypertension.

What Is Relevant?

- These studies give a better understanding of sympathetic activation in renovascular hypertension.

Summary

The activity of Ang-(1–7) and Mas receptors in RVL contributes to the enhanced CSAR and sympathetic activation, and the cAMP-PKA pathway is involved in Ang-(1–7)–mediated effects in renovascular hypertension.
Angiotensin-(1–7) in the Rostral Ventrolateral Medulla Modulates Enhanced Cardiac Sympathetic Afferent Reflex and Sympathetic Activation in Renovascular Hypertensive Rats

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Angiotensin-(1-7) in the Rostral Ventrolateral Medulla modulates enhanced Cardiac Sympathetic Afferent Reflex and Sympathetic Activation in Renovascular Hypertensive Rats

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Supplemental Methods

Renovascular hypertensive model

Goldblatt’s two-kidney, one-clip (2K1C) method was used to elicit renovascular hypertension as in our previous reports. 1-3 Briefly, a retroperitoneal flank incision was performed in rats weighing 160-180 g under anesthesia with intraperitoneal sodium pentobarbital administration (50 mg kg⁻¹). The right renal artery was exposed and partly occluded by placing a U-shaped silver clip with a 0.20 mm internal diameter on the artery to induce renovascular hypertension. Normotensive sham-operated (Sham) rats received a similar surgical process, except without a silver clip. The rats were kept in a temperature-controlled room on a 12 h–12 h light–dark cycle with free access to standard chow and tap water. Acute experiments were performed at the end of the 4th week after the surgery. The criterion for hypertension in the present study was determined to be systolic blood pressure (SBP) >160 mm Hg. 1-3 Only rats with SBP > 160 mm Hg underwent acute experiments in the 2K1C rat group. Ten rats were excluded because SBP in these rats was not high enough to meet this criterion.

SBP measurements

The tail artery SBP was measured in conscious rats with a noninvasive computerized tail-cuff system (NIBP, ADInstruments, Australia). 1-3 To minimize stress-induced SBP fluctuations, the rats were trained by measuring SBP daily for at least 10 days before the 2K1C or sham operations and at weekly intervals throughout the 4-week post-2K1C or sham operation period. The rats were warmed for 10-20 min at 28 °C before the measurements to allow for detection of tail arterial pulsations and to achieve the steady pulse. The SBP was obtained by averaging 10 measurements.

Echocardiography measurements

Rat transthoracic echocardiography was performed 4 weeks after the 2K1C or sham operations under light anesthesia (50 mg kg⁻¹ sodium pentobarbital, i.p.) with an ultrasound system (Vevo 2100, VisualSonics, Canada) using a 21-MHz probe to determine left ventricular function. The left ventricular end-diastolic diameter (LVEDD), end-systolic diameter (LVESD), interventricular septal thickness in diastole (IVSd) and systole (IVSs) as well as the left ventricular posterior wall thickness in diastole (LVPWd) and systole (LVPWs) were measured. The left ventricular (LV) fractional shortening (FS), ejection fraction (EF), LV mass and LV mass/body weight (BW) were calculated. All measures were averaged over three consecutive cardiac cycles.

General procedures of the acute experiment

Each rat was intraperitoneally anesthetized with urethane (800 mg kg⁻¹) and α-chloralose (40 mg kg⁻¹) at the end of the 4th week after 2K1C or Sham surgery. Supplemental anesthesia doses were used to maintain an appropriate level of anesthesia, which was assessed by the absence of corneal reflexes and a paw withdrawal response to a noxious pinch. The rat was mechanically ventilated with
room air using a rodent ventilator (model 683, Harvard Apparatus Inc., USA). The right carotid artery was cannulated and connected with a pressure transducer (MLT0380, ADInstruments, Australia) for continuous arterial blood pressure (ABP), MAP and heart rate (HR) recording. Bilateral baroreceptor denervation and vagotomy were performed and identified as previously reported. 4,5

**RSNA recordings**

A retroperitoneal incision was performed, and the left renal sympathetic nerve was isolated. The renal nerve was cut distally to eliminate its afferent activity. The nerve was placed on a pair of silver electrodes and immersed in warm mineral oil. Nerve signals were amplified with a four channel AC/DC differential amplifier (DP-304, Warner Instruments, Hamden, CT, USA) with a high pass filter at 10 Hz and a low pass filter at 3,000 Hz. The RSNA was integrated at a time constant of 100 ms. At the end of each experiment, background noise was determined after sectioning of the central end of the nerve 6 and was subtracted from the integrated RSNA values. Raw and integrated RSNA, ABP, MAP and HR were simultaneously recorded with a PowerLab data acquisition system (8SP, ADInstruments, Australia).

**Evaluation of the CSAR**

A limited left lateral thoracotomy was performed to expose the heart, and the pericardium was removed. The CSAR was induced by stimulating cardiac sympathetic afferents by applying a piece of filter paper (3 mm × 3 mm) containing capsaicin (1.0 nmol in 2.0 μl) to the epicardium on the anterior wall of the left ventricle. One minute later, the filter paper was removed, and the ventricular surface was rinsed three times with 10 ml normal saline (38 °C). The CSAR was evaluated by the RSNA and MAP responses to the epicardial capsaicin application. 7,8

**RVLM microinjection**

The rats were placed in a stereotaxic frame (Stoelting, Chicago, USA). The RVLM coordinates were 4.5-5.0 mm posterior to lambda, 2.0-2.3 mm lateral to midline and 8.1-8.4 mm below the dorsal surface of the cerebellum. 7,8 Bilateral RVLM microinjections were performed with two glass micropipettes (50 μm tip diameter) and the injection rate was controlled by a dual-channel microdialysis infusion syringe pump (53101V, Stoelting Co., Illinois, USA). The microinjection volume was 50 nL for each side of the RVLM, and the microinjection was completed within 1 min. The functional RVLM location was assessed by a pressor response of more than 25 mm Hg to a glutamate (2 nmol) microinjection. At the end of the experiment, 50 nL of 2% Evans Blue dye was injected into each microinjection site, except for those in which the Ang-(1-7) and cAMP levels were measured, so as not to interfere with the ELISA results. The microinjection sites were histologically verified with microscopy. In total, 25 rats with microinjection sites outside of the RVLM were excluded from data analysis. A representative photograph of the RVLM microinjection sites is shown in online-only Data Supplement Figure S1.

**Mas receptor immunohistochemistry**
Mas receptor immunohistochemistry in the RVLM was performed with an immunohistochemistry kit (Abcam, HKSP, New Territories, HK). Briefly, the brains were processed as our previous report and coronal brain sections (25 μm) at the RVLM level were incubated with rabbit polyclonal Mas receptor antibodies (1:200, Alomone Labs, Israel) at 4 °C overnight. After washing, sections were incubated with biotinylated goat anti rabbit IgG for 10 min and then stained with DAB according to the manufacturer’s instructions. Sections were covered with mounting medium and Mas receptor immunoreactivity was observed under a light microscope (DP70, Olympus, Tokyo, Japan).

RVLM Sample Preparation

Rats were euthanized with a pentobarbital overdose. The brain was quickly removed, frozen with liquid nitrogen, and stored at -70 °C until sectioning. Coronal brain sections were made with a cryostat microtome (Leica CM1900-1-1, Wetzlar, Hessen, Germany) at the RVLM level, and the RVLM area was removed with a 15-gauge needle (1.5 mm ID). The removed tissues were subsequently homogenized and centrifuged. Total protein in the homogenate supernatant was extracted and measured by using a protein assay kit (BCA, Pierce).

Mas receptor protein expression measurements

Mas receptor protein expression in the RVLM was determined by Western blotting. Briefly, after process of electrophoresis and transmembrane, proteins on nitrocellulose membrane were probed with rabbit polyclonal Mas receptor antibodies (1:200, Alomone Labs, Israel) followed by incubation with horseradish peroxidase–conjugated goat anti-rabbit IgG (1:5000; Immunology Consultants Lab, USA). The bands were visualized by enhanced chemiluminescence using ECL (Pierce Chemical), and GAPDH (Bioworld Technology Inc., USA) protein was used as a loading control. The total Mas receptor protein level was normalized to the GAPDH protein level.

Ang-(1-7) level measurements

The Ang-(1-7) level in the RVLM tissue homogenate was measured using a commercial ELISA kit (MyBioSource LLC, USA) following the manufacturer’s instructions.

cAMP level measurements

The cAMP level in the RVLM was determined using a commercial ELISA kit (Cayman Chemical Co, USA) following the manufacturer’s instructions.

Experimental design

Acute experiments were performed at the end of the 4th week after the clipping or sham surgery. First, rats were kept in a supine position. The trachea and right carotid artery were cannulated and connected with a rodent ventilator and a pressure transducer respectively, and the sinoaortic denervation and vagotomy were performed.
Then, the rats were kept in a prone position with the head fixed in the stereotaxic frame for RVLM positioning. Finally, the rat body was kept in the right lateral position with the head fixed in the stereotaxic frame, and its left foreleg was placed behind its body to expose its left thorax. A left lateral thoracotomy was performed to prepare for epicardial chemical application. A left retroperitoneal incision was made to place RSNA recording electrodes. The rats were stabilized for approximately 30 min before intervention.

**Experiment 1:** The effects of Ang-(1-7) and the Mas receptor antagonist A-779 in the RVLM on RSNA, MAP and CSAR were determined. Both Sham and 2K1C rats were randomly divided into 8 groups (n=6 per group) that were subjected to the RVLM microinjection of saline, three doses of Ang-(1-7) (0.03, 0.3 and 3 nmol), three doses of A-779 (0.03, 0.3 and 3 nmol) and Ang-(1-7) (3 nmol) pretreated with A-779 (3 nmol). In addition, to determine the binding specificity of A-779 to the Mas receptor, RVLM microinjection of Ang II (3 nmol) and pretreatment with A-779 (3 nmol) before Ang II were performed in another 2 groups of Sham and 2K1C rats. Pretreatment with A-779 occurred 8 min before Ang-(1-7) or Ang II treatment. RVLM microinjection-induced baseline RSNA and MAP changes were determined by averaging 2 min of the maximal responses. The RSNA change was expressed as the percent change from the values before RVLM microinjection. The CSAR was evaluated by the RSNA and MAP responses to epicardial capsaicin (1 nmol) application 8 min after the Ang-(1-7) or A-779 microinjection or 3 min after the Ang II microinjection. The RSNA and MAP responses to capsaicin application were determined 15 sec after the capsaicin by averaging the parameters for 30 sec.

**Experiment 2:** The Ang-(1-7) level, Mas receptor protein expression and immunohistochemistry in the RVLM were assessed in 2K1C rats compared with Sham rats. Three groups of 2K1C rats or Sham rats (n=5 per group) were euthanized, and one group was used to measure Ang-(1-7) level, the second group was used to determine Mas receptor protein expression by Western blotting, and the third group was used to perform Mas receptor immunohistochemistry.

**Experiment 3:** Both Sham and 2K1C rats were randomly divided into 3 groups (n=7 per group) that were respectively subjected to the RVLM microinjection of saline, Ang-(1-7) (3 nmol) and A-779 (3 nmol). Echocardiography was performed 8 min after the RVLM microinjection.

**Experiment 4:** The roles of cAMP and PKA in RVLM in regulating RSNA, MAP and CSAR as well as the effects of Ang-(1-7) were determined. The effects of RVLM microinjection of saline, SQ22536 (2 nmol, an AC inhibitor), db-cAMP (1 nmol, a cAMP analogue), Rp-cAMP (1 nmol, a PKA inhibitor), 1% DMSO, MDL-12,330A (2 nmol, another AC inhibitor) and H-89 (2 nmol, another PKA inhibitor) on RSNA, MAP and CSAR were assessed in 7 groups of Sham rats and 7 groups of 2K1C rats (n=6 per group). The CSAR was evaluated 8 min after RVLM microinjection. In another 7 groups of Sham and 2K1C rats, the effects of RVLM pretreatment with the same doses of saline, SQ22536, db-cAMP, Rp-cAMP, DMSO, MDL-12,330A and
H-89 as above on Ang-(1-7) (3 nmol) induced RSNA, MAP and CSAR responses were determined (n=6 per group). The pretreatment was performed 8 min before Ang-(1-7) treatment, and the CSAR was evaluated 8 min after Ang-(1-7).

**Experiment 5:** The effects of RVLM microinjection of saline, Ang-(1-7) (3 nmol), A-779 (3 nmol) and epicardial application of saline or capsaicin on cAMP level in RVLM were determined in 5 groups of Sham and 5 groups of 2K1C rats (n=5 per group). The effects of RVLM pretreatment with saline, Ang-(1-7) and A-779 on epicardial capsaicin application-induced changes in cAMP level were determined in another 3 groups of Sham and 2K1C rats (n=5 per group). RVLM pretreatment was performed 8 min before the epicardial application. Rats were decapitated 8 min after the RVLM microinjection or 1 min after the epicardial application, and the brains were prepared for cAMP level measurement.

**Reference**


Supplemental Tables

**Table S1.** The body weight, systolic blood pressure (SBP), baseline mean arterial pressure (MAP) and baseline heart rate (HR) at the end of the 4th week after two-kidney, one-clip (2K1C) surgery or sham operation.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Sham</th>
<th>2K1C</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>199</td>
<td>199</td>
</tr>
<tr>
<td>Body Weight, g</td>
<td>326.7 ± 2.1</td>
<td>324.1 ± 2.3</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>119.6 ± 2.1</td>
<td>191.7 ± 3.0*</td>
</tr>
<tr>
<td>Baseline MAP, mm Hg</td>
<td>90.6 ± 1.5</td>
<td>126.9 ± 1.8*</td>
</tr>
<tr>
<td>Baseline HR, beats/min</td>
<td>340.7 ± 4.2</td>
<td>350.8 ± 5.1</td>
</tr>
</tbody>
</table>

The SBP of tail artery was measured in conscious rats using a noninvasive computerized tail-cuff system. The baseline MAP and HR were measured under anesthesia with a pressure transducer through a catheter placed in the right carotid artery. Values are expressed as mean ± SE. * P<0.05 compared with the sham-operated (Sham) rats.
Table S2. Echocardiographic data of the left ventricle in sham-operated (Sham) rats and two-kidney, one-clip (2K1C) rats.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Sham</th>
<th>2K1C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Ang-(1-7)</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>6.72 ± 0.22</td>
<td>6.80 ± 0.29</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>3.86 ± 0.13</td>
<td>3.96 ± 0.14</td>
</tr>
<tr>
<td>IVSd, mm</td>
<td>1.55 ± 0.04</td>
<td>1.46 ± 0.02</td>
</tr>
<tr>
<td>IVSs, mm</td>
<td>2.69 ± 0.11</td>
<td>2.42 ± 0.06</td>
</tr>
<tr>
<td>LVPWd, mm</td>
<td>1.82 ± 0.03</td>
<td>1.77 ± 0.05</td>
</tr>
<tr>
<td>LVPWs, mm</td>
<td>2.76 ± 0.05</td>
<td>2.77 ± 0.08</td>
</tr>
<tr>
<td>FS, %</td>
<td>43.3 ± 1.87</td>
<td>42.1 ± 1.25</td>
</tr>
<tr>
<td>EF, %</td>
<td>72.9 ± 1.99</td>
<td>72.7 ± 0.97</td>
</tr>
<tr>
<td>LV mass (g)</td>
<td>0.76 ± 0.03</td>
<td>0.73 ± 0.04</td>
</tr>
<tr>
<td>LV mass/BW (mg/g)</td>
<td>2.34 ± 0.09</td>
<td>2.24 ± 0.11</td>
</tr>
</tbody>
</table>

Ang-(1-7) angiotensin (Ang)-(1-7), A-779 D-Alanine-Ang-(1 -7), LVEDD left ventricular end-diastolic diameter, LVESD left ventricular end-systolic diameter, IVSd interventricular septal thickness in diastole, IVSs interventricular septal thickness in systole, LVPWd left ventricular posterior wall thickness in diastole, LVPWs left ventricular posterior wall thickness in systole, FS fractional shortening, EF ejection fraction, LV left ventricular, BW body weight.

Values are mean ± SE. * P<0.05 compared with Sham. n=7 for each group.
Supplemental Figures

Figure S1. Representative brain slice showing the microinjection sites in rostral ventrolateral medulla (RVLM). The arrows point to the microinjection sites.
Figure S2. Representative recordings showing the effects of rostral ventrolateral medulla (RVLM) microinjection of saline and angiotensin (Ang)-(1-7) (3 nmol) on the cardiac sympathetic afferent reflex (CSAR) in sham-operated (Sham) and two-kidney, one-clip (2K1C) rats. The CSAR was evaluated by the renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP) responses to epicardial capsaicin application. RVLM microinjection of the Ang-(1-7) significantly enhanced the CSAR more in 2K1C rats than in Sham rats.
Figure S3. Representative photos showing the Mas receptor immunohistochemistry in the rostral ventrolateral medulla (RVLM) in sham-operated (Sham) and two-kidney, one-clip (2K1C) rats. Mas receptor expression in RVLM in 2K1C rats was increased compared with Sham rats.