Apelin Gene Transfer Into the Rostral Ventrolateral Medulla Induces Chronic Blood Pressure Elevation in Normotensive Rats

Qi Zhang, Fanrong Yao, Mohan K. Raizada, Stephen T. O’Rourke, Chengwen Sun

Abstract—The peripheral apelin system plays a significant role in cardiovascular homeostasis and in the pathophysiology of cardiovascular diseases. However, the central effect of this neurohormonal system in neural control of cardiovascular function remains poorly understood. Thus, this study was undertaken to evaluate the effect of apelin in the rostral ventrolateral medulla (RVLM) on blood pressure, cardiac function, and sympathetic nerve activity. Apelin mRNA and protein levels were detected with real-time RT-PCR and Western blots, respectively. Expression of apelin was significantly enhanced in the RVLM of spontaneously hypertensive rat (SHR) compared with normotensive Wistar–Kyoto (WKY) rats. To study the functional consequence of upregulated apelin expression, apelin was overexpressed by bilateral microinjection of the AAV2-apelin viral vector into the RVLM of WKY rats. Immunofluorescence staining and Western blots demonstrated that microinjection of AAV2-apelin into the RVLM resulted in a significant increase in apelin expression, which was associated with a chronic elevation in blood pressure and cardiac hypertrophy. In addition, direct microinjection of exogenous apelin-13 (200 pmol in 50 nL) into the RVLM caused a 20 mm Hg elevation in blood pressure and a 24% increase in sympathetic nerve activity. The present study is the first to show that apelin expression is enhanced in the RVLM of SHR versus WKY rats and that overexpression of this gene in the RVLM results in chronic blood pressure elevation and cardiac hypertrophy in normotensive rats. Thus, the apelin system in the RVLM may play a very important role in central blood pressure regulation and in the pathogenesis of hypertension. (Circ Res. 2009;104:1421-1428.)

Key Words: blood pressure ■ hypertension ■ hypertrophy ■ brain ■ sympathetic

Apelin is a peptide recently isolated from bovine stomach extracts.1 It has been identified as the endogenous ligand for the orphan G protein–coupled receptor APJ,1,2 which is comprised of 7 transmembrane domains and shares a 31% amino acid sequence identity to the angiotensin II type 1 receptor.3 Despite the degree of homology between these receptors, angiotensin (Ang) II does not bind the APJ receptor, and apelin is the only known ligand for the APJ receptor.3 Multiple apelin peptides appear to be derived from a 77-aa precursor peptide, including preproapelin apelin-36 (42-77), apelin-17 (61-77), and apelin-13 (65-77). Apelin and APJ are widely distributed in various tissues and are thought to be involved in cardiovascular regulation,4,5 heart contractility, body fluid homeostasis,6 control of appetite, and, possibly, immune functions.7 The role of apelin and APJ in the cardiovascular system is currently the best documented. The accumulated evidence indicates that apelin and its APJ receptor are expressed throughout the cardiovascular system. Both pressor and depressor responses have been described in response to peripheral administration of apelin.1,5,8–10 Genetic studies in humans also demonstrate that disruption of the endogenous apelin/APJ system may have functional relevance in human heart failure. In these patients, the presence of a polymorphism of the APJ receptor 212A was associated with slower progression of heart failure.11 In addition, a single-nucleotide polymorphism in the APJ gene is associated with increased susceptibility to brain infarction.12 Thus, the emerging evidence indicates that the apelin/APJ system plays a significant role in cardiovascular homeostasis and in the pathophysiology of cardiovascular diseases. However, the involvement of this neurohormonal system in neural control of cardiovascular function remains poorly understood.

In the brain, apelin-immunoreactive cell bodies and fibers and mRNA for apelin and the APJ receptor are distributed predominantly in neurons of the hypothalamus and brain stem, including cardiovascular regulatory regions such as the paraventricular nucleus, supraoptic nucleus, circumventricular organs, nucleus tractus solitarius (NTS), and rostral ventrolateral medulla (RVLM).2,13–15 The RVLM is a key integrative site within the medulla that participates in the
tonic and baroreflex regulation of blood pressure (BP) via sympathetic nerve activity (SNA). “Pacemaker cells” within this area provide the major excitatory input to sympathetic preganglionic neurons in the spinal cord that innervate sympathetic ganglia and the adrenal medulla.\textsuperscript{16,17} In addition, the RVLM receives extensive inputs from other cardiovascular nuclei, including tonic excitatory input from the paraventricular nucleus and NTS and inhibitory input from the caudal ventrolateral medulla.\textsuperscript{18,19} Thus, the RVLM is considered the major relay point for the transmission of SNA. In addition, both altered RVLM function and elevated SNA have been implicated in the pathogenesis of hypertension in several hypertensive animal models.\textsuperscript{20–23}

The objectives of the present study were 2-fold: (1) to compare apelin expression in the RVLM between spontaneously hypertensive rats (SHR) and normotensive Wistar–Kyoto (WKY) rats; and (2) to determine the effects of endogenous and exogenous apelin in the RVLM on BP and SNA in vivo. Our results indicate that apelin expression is markedly enhanced in the RVLM of SHR compared with WKY rats; moreover, overexpression of apelin in the RVLM induces chronic elevation of BP and significant cardiac hypertrophy in normotensive WKY rats. We also demonstrate that exogenous apelin-13, microinjected into the RVLM, causes an acute increase in BP and an elevation in renal (R)SNA.

Materials and Methods

Animals

Adult male SHR and WKY rats (9 to 10 weeks old) were obtained from Charles River Farms (Wilmington, Mass). Rats were housed individually and kept on a 12:12-hour light/dark cycle in a climate-controlled room. Rat chow (Harlan Tekland, Madison, Wis) and water were provided ad libitum. All experimental procedures were approved by the North Dakota State University Institutional Animal Care and Use Committee (protocol A0743).

Real-Time RT-PCR

Apelin mRNA levels in the RVLM of SHR and WKY rats were determined by real time RT-PCR, as described in our previous study.\textsuperscript{24} TaqMan probes specific for rat apelin were purchased from Applied Biosystems Inc (Foster City, Calif). Real-time RT-PCR was performed in an Applied Biosystems PRISM 7000 sequence detection system according to the protocol from the manufacturer. Data were normalized to 18S RNA. In each experiment, samples were analyzed in triplicate.

Western Blot Analysis

Apelin protein levels in the RVLM, NTS, and area postrema of SHR and WKY rats were assessed by Western blot analysis, as described previously.\textsuperscript{24} with a primary antibody, rabbit anti-apelin antibody (Santa Cruz Biotechnology Inc, Santa Cruz, Calif; dilution, 1:500), and a secondary antibody, anti-rabbit peroxidase-conjugated antibody (Bio-Rad, Hercules, Calif; dilution, 1:15 000). The primary rabbit anti-apelin antibody is raised against animo acids 38 to 77 at the C terminus of apelin. Thus, it can identify preproapelin (apelin-36), apelin-28, and apelin-13. Immunoreactivity was detected by enhanced chemiluminescence autoradiography (ECL Western blotting detection kit, Amersham Pharmacia Biotechnology), and film was analyzed using Quantity One Software (Bio-Rad).

Immunofluorescence Staining

The immunofluorescence staining of brain RVLM sections was performed as we have described previously.\textsuperscript{24} In brief, the sections were incubated with primary antibodies (rabbit anti-apelin antibody and anti-NeuN monoclonal antibody; dilution, 1:500) overnight at 4°C. On the next day, the sections were washed and incubated with secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG 1:1000 and Alexa Fluor 594 goat anti-mouse IgG 1:1000, Molecular Probes, Carlsbad, Calif) for 2 hours at room temperature. The sections were then washed with PBS/Tween and mounted with antibleaching medium on a glass coverslip. The fluorescence staining was detected using an Olympus fluorescence microscope equipped with a color digital camera (Infinity 2), which was connected to a computer to capture and analyze images using Infinity Capture and Analyze Software.

RVL M Microinjection and Apelin Gene Transfer Into the RVLM

Male WKY rats (9 to 10 weeks old) were anesthetized with a mixture of O\textsubscript{2} (1 L/min), and isoflurane (3%) was delivered through a nose cone. The rats were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, Calif). Skin overlying the midline of the skull was incised, and a small hole was drilled bilaterally on the dorsal surface of the cranium according to the following coordinates: 1.9 mm lateral to the midline, 3.0 mm posterior to the lambdoid suture, 10 mm below the skull. Bilateral RVLM microinjections (50 nL) of either apelin-13 or saline were performed to examine the effect of exogenous apelin. In addition, an adeno-associated virus type 2 (AAV2) containing the rat apelin gene (AAV2-apelin, with AAV2–green fluorescent protein [AAV2-GFP] as the control virus), driven by a chicken β-actin promoter with a human cytomegalovirus enhancer, were used to induce endogenous apelin expression in the RVLM. AAV2-GFP and AAV2-apelin plasmids were constructed and prepared as described previously.\textsuperscript{25} The AAV2-apelin or AAV2-GFP (all in 1×10\textsuperscript{6} genome copy in 50 nL) was microinjected bilaterally into the RVLM over a 25-minute period with a microinjection device. The RVLM location of the microinjection site was verified by the pressor response to L-glutamate microinjection as described in our previous publication.\textsuperscript{26}

Abdominal Aorta Cannulation

Animals were anesthetized with inhaled isoflurane as described above. Radiotelemetric pressure transducers (Data Sciences International, St Paul, Minn) consisting of a fluid-filled catheter attached to a PA-C40 transmitter were implanted into the abdominal aorta as described previously.\textsuperscript{27} Before implantation, the aorta was clamped proximally and the catheter inserted and secured with medical adhesive. After BP transducer implantation, rats were left to recover for a week. BP and heart rate (HR) were recorded in conscious rats from 10:00 to 11:00 AM, once a day, using Dataquest IV software (Data Sciences International). The basal BP and HR were recorded for 3 days before RVLM microinjection of AAV2-apelin or AAV2-GFP as described above.

Assessment of Cardiac Hypertrophy

Fourteen days after RVLM gene transfer, the animals were anesthetized with sodium pentobarbital and the hearts were removed. After the heart weight and cardiac morphology were examined, hearts were fixed in 10% formalin/PBS and embedded in paraffin. Heart sections (10 μm) were stained with hematoxylin/eosin to evaluate morphology and cellular dimensions.

Assessment of Sympathetic Nerve Activity and BP

Male WKY rats (9 to 10 weeks old) were anesthetized with isoflurane, as described above, and PE-10 catheters fused to PE-50 catheters were prefilled with heparinized saline (100 IU/mL) and placed in the right femoral artery for acute recording of BP and HR. The left kidney was exposed via a left flank incision and one of the renal nerves was dissected, hooked to a pair of silver electrodes, and glued together with SilGel 604 (Wacker, Munich, Germany). The electrodes were connected to an EX1 Differential Amplifier. BP and HR were recorded using a pressure transducer, which was connected
to a Bridge Amplifier (AD Instrument, Colorado Springs, Colo). Both BP and RSNA data were collected and analyzed with PowerLab software (AD Instruments).

**Statistical Analyses**

All data are presented as means±SE. Statistical significance was evaluated by 1- or 2-way ANOVA, as appropriate, followed by either a Newman–Keuls or Bonferroni post hoc analysis where appropriate. Differences were considered significant at $P<0.05$, and individual probability values are noted in the figure legends.

**Results**

**Expression of Apelin in the RVLM of SHR Versus WKY Rat**

Apelin protein levels in the RVLM of SHR and WKY rats were determined by Western blot analysis and revealed that apelin protein levels were 40% higher in the RVLM of SHR as compared with WKY rats (Figure 1A and 1C; $n=4$ in each strain). As a control, we also determined apelin protein levels in other cardiovascular regulatory regions in the brain stem of SHR and WKY rats, including NTS and area postrema, using Western blot analysis. Apelin protein levels in these 2 brain areas were comparable between SHR and WKY rats (Figure 1B and 1C; $n=4$ in each strain). In addition, apelin mRNA levels were determined in micropunches of RVLM from SHR and WKY rats using real-time RT-PCR. Data in Figure 1D demonstrate that apelin is expressed in the RVLM of both SHR and WKY rats and that the mRNA levels of apelin are enhanced in the RVLM of SHR as compared with WKY rats ($n=4$ in each strain). Taken together, these observations

![Figure 1](image1.png)

*Figure 1.* Expression of apelin is enhanced in the RVLM of SHR rats. A, Representative autoradiogram of apelin protein levels in the RVLM: Western blot analysis was used to measure apelin levels from 20 μg of total cell lysate isolated from RVLM micropunches of SHR and WKY rats. Data are normalized using β-actin. B, Representative autoradiogram of apelin protein (AP) levels in the NTS and area postrema of SHRs and WKY rats as described for A. C, Bar graphs summarizing the quantitation of the apelin protein bands. Data are means±SE of apelin vs β-actin protein in the RVLM, NTS, or AP of SHR or WKY rats ($n=4$ in each strain). *P<0.05 compared with apelin protein levels in the RVLM of WKY rats. D, Bar graphs showing the mRNA levels of apelin in the RVLM of SHR and WKY rats. The apelin mRNA levels were detected using real-time RT-PCR. Data are means±SE from SHR and WKY rats ($n=4$ in each strain).

![Figure 2](image2.png)

*Figure 2.* Overexpression of apelin in the RVLM of WKY rats. A through D, Immunofluorescence images showing overexpression of apelin protein in the RVLM of rats after 7 days of apelin gene transfer. A, Fluorescence micrograph (×40 magnification) demonstrating localization of apelin in RVLM cells, immunostained with anti-apelin antibody (green). B, Same field of cells as in A, immunostained with anti-NeuN antibodies (red). C, Overlap of A and B, showing the green fluorescence is neuronally located. D, Fluorescence micrograph (×10 magnification) demonstrating localization of apelin within the RVLM, indicated by the square in E. 7 days after injection of AAV2-apelin (50 nL of 1×10^11 gc). E, Location of the stained RVLM brain sections shown in A through D, based on the rat brain atlas of Swanson. SPVI indicates spinal tract of the trigeminal interpolar part; PY, pyramid. Scale bars=50 μm.
demonstrate that apelin expression is enhanced in the RVLM of SHR as compared with WKY rats.

Overexpression of Apelin in the RVLM by Gene Transfer

To study the functional consequence of enhanced apelin expression in the SHR RVLM on BP regulation, the apelin gene was overexpressed using AAV2 viral vector-mediated gene transfer in the RVLM of normotensive WKY rats. Figure 2 shows immunofluorescence of apelin expression in the RVLM on the seventh day after microinjection of AAV2-apelin. Figure 2A through 2C shows a high magnification view indicating that apelin expression is localized to neurons of the RVLM. Figure 2D and 2E demonstrates a strong immunoreactive apelin signal in the RVLM after transfection. The time course of apelin protein expression in the RVLM after AAV2-apelin or AAV2-GFP transfection is shown in Figure 3A and 3B. A representative Western blot showing apelin protein levels in the RVLM at various time points after gene transfer is shown in Figure 3A, and bar graph summaries of the quantified group data are shown in Figure 3B. Compared with the control rats, AAV2-mediated apelin gene transfer induced a significant increase in apelin protein expression within 24 hours (ratio of apelin:β-actin: 0.15±0.02 in control and 0.38±0.05 at 24 hours after gene transfer; n=4 rats; P<0.01). On day 2 after gene transfer, apelin protein expression reached its peak (0.68±0.05) and was sustained for at least 14 days (0.79±0.06). However, microinjection of AAV2-GFP control vector into the RVLM did not alter apelin protein levels (0.15±0.02 in control rats and 0.16±0.04 at 14 day after gene transfer; n=4 rats; P>0.05).

Effect of Gene Transfer on Arterial Pressure and Cardiac Hypertrophy

Based on the above apelin expression data, we next examined whether increased expression of apelin in the RVLM of normotensive rats would alter basal BP. Nine-week-old WKY rats were fitted with telemetry pressure transducers, followed 1 week later by microinjection of either AAV2-apelin (1×10^9 gc in 50 nL) or AAV2-GFP (1×10^9 gc in 50 nL) bilaterally into the RVLM. Mean arterial pressure (MAP) and HR were monitored via telemetry before AAV2 vector injections into the RVLM and for 14 days after viral vector injections (Figure 3C). Before the RVLM microinjections, there was no difference in basal MAP between the AAV2-apelin- and AAV2-GFP treated groups. After gene transfer, MAP was significantly elevated compared to control MAP (P<0.05).
AAV2-GFP–treated WKY rats (93.2±5.1 mm Hg in AAV2-apelin group and 88±3.9 mm Hg in AAV2-GFP group; n=7 and n=6, respectively; P>0.05). After RVLM microinjection of AAV2-apelin, the MAP increased steadily, reaching a peak level of 121±5.4 mm Hg 3 days after microinjection and remained elevated throughout the 14-day recording period. In contrast, microinjection of AAV2-GFP into the RVLM of WKY rats did not alter the basal MAP. The effect of AAV2-apelin and AAV2-GFP microinjected into the RVLM on HR is shown in Figure 3D, indicating that neither AAV2-Apelin nor AAV2-GFP had any significant effect on HR in WKY rats. In addition, cardiac morphology was compared between the rats receiving microinjection of AAV2-GFP and AAV2-apelin into the RVLM. The body weight was not significantly different between the 2 groups of rats (395±11 g in AAV2-GFP group and 395±6 g in AAV2-apelin group). However, the heart weight/body weight ratio and the myocyte cross sectional area were significantly increased by microinjection of AAV2-apelin into the RVLM (Figure 4). Taken together, these results indicate that overexpression of apelin in the RVLM results in chronic elevation in BP and a remarkable cardiac hypertrophy, without altering HR.

Effects of Exogenous Apelin-13 Microinjected Into the RVLM on BP and RSNA

To determine whether elevated apelin-13 levels in the RVLM cause BP elevation and sympathetic nervous system activation, exogenous apelin-13 was microinjected into the RVLM of WKY rats. MAP and RSNA were recorded before and after apelin-13 microinjection into the RVLM. Baseline MAP and HR were similar between the apelin group and saline group (Figure 5B). Microinjection of apelin-13 (200 pmol in 50 nL) into the RVLM induced a significant MAP elevation (from 95.9±1.1 mm Hg to 115.5±2.5 mm Hg; n=7; P<0.01; Figure 5A and 5B). The time-dependent pressor effect of apelin microinjection started at 2 minutes, peaked at 4 to 10 minutes, and lasted ~14 minutes after apelin injection (Figure 5B). However, RVLM microinjection of saline (50 nL) control did not alter arterial pressure (Figure 5B). In addition, apelin-13 microinjected into RVLM also resulted in a significant increase in HR (from 339±5 bpm to 378±5 bpm; n=7; P<0.01; Figure 5C). In contrast, RVLM microinjection of saline (50 nL) control had no effect on the basal HR (Figure 5C).

To determine whether the pressor effect of apelin-13 in the RVLM is associated with an alteration of sympathetic nerve activation, RSNA was recorded during apelin microinjection. Microinjection of apelin-13 (200 pmol in 50 nL) into the RVLM increased RSNA by 24±6% in 7 WKY rats (Figure 5D and 5E); however, injection of saline control (50 nL) into the RVLM did not significantly alter the basal activity of sympathetic outflow (Figure 5D and 5E).

Discussion

The present studies present the first evidence that apelin expression is increased in the RVLM of SHR and that viral vector-mediated overexpression of apelin in the RVLM induces a chronic elevation in BP in normotensive WKY rats. These results, coupled with the observation that exogenous microinjection of apelin-13 into the RVLM increases BP and RSNA, strongly suggest that the apelin/APJ system is impor-
tant in centrally mediated neural control of the cardiovascular system.

It is well documented that apelin is distributed throughout the medulla oblongata, including the RVLM, but the functional role of the peptide has not yet been clarified. In the present study, we compared apelin expression in the RVLM of SHR and WKY rats and demonstrated that both mRNA and protein levels of apelin are markedly increased in the RVLM of hypertensive rats. To determine whether the upregulated expression of apelin observed in the RVLM of SHR is able to drive BP to a higher level, we transferred the apelin gene into the RVLM of normotensive WKY rats using an AAV2 vector. Microinjection of AAV2-apelin into the RVLM successfully induced a chronic overexpression of apelin. Moreover, in conscious WKY rats, overexpression of apelin by the AAV2 vector-mediated gene transfer system resulted in a significant and sustained increase in mean arterial BP, as well as cardiac hypertrophy. Under these chronic conditions, HR was not significantly altered by AAV2-apelin gene transfer. The mechanism of this phenomenon is unknown but could be mediated by activation of a neuronal circuit or signaling pathways, which attenuate the apelin-induced tachycardia observed in the acute experiments of the present study.

It has been reported by Seyedabadi et al that microinjection of apelin-13 into the RVLM increases arterial pressure and phrenic nerve discharge amplitude. Consistent with these observations, the present study demonstrated that acute microinjection of exogenous apelin-13 into the RVLM increases BP and that the pressor effect of apelin-13 injected into the RVLM is associated with an elevation in RSNA. Considerable evidence indicates that increased sympathetic nervous system activity appears to be a critical mechanism, not only in hypertension, but also in the development of target organ pathologies such as cardiac and vascular hypertrophy, and renal failure. For example, morbidity and mortality in cardiac failure are linked to neurohumoral excitation and increased sympathetic drive. Sympathetic hyperactivity is also implicated in several metabolic disorders, such as diabetes and obesity, which are well-known risk factors for cardiovascular diseases. Thus, the excitatory effect of apelin on sympathetic outflow suggests that the apelin/APJ pathway plays a crucial role in the pathogenesis of hypertension and other cardiovascular diseases.

Figure 5. Effect of apelin-13 injected into the RVLM on BP, HR, and RSNA. A, Representative tracing showing an arterial BP recording before and after microinjection of apelin-13 into the RVLM of WKY rats. Arrow indicates the microinjection of apelin-13. B, Time course showing the effect of apelin-13 (200 pmol, 50 nL) or saline control (50 nL) microinjected into the RVLM on MAP. Data are means±SE (n=5 or n=7 rats in each group) of MAP recorded every minute before and after RVLM injection of apelin-13 or saline control. *P<0.05 vs saline control group. C, Time course of HR change before and after RVLM microinjection of apelin-13 (200 pmol, 50 nL) or saline control (50 nL). Data are means±SE (n=5 to 7 rats in each group). *P<0.05 vs saline control group, **P<0.01 vs saline control group. D, Representative recording of RSNA and integrated RSNA (fRSNA) in response to exogenous apelin-13 (200 pmol in 50 nL) injected into the RVLM of WKY rats. Arrow indicates the microinjection of apelin-13. E, Bar graphs summarizing the effect of apelin-13 (200 pmol in 50 nL) or saline control (50 nL) microinjected into the RVLM on RSNA in WKY rats. Data are means±SE (n=5 to 7 rats). *P<0.05 compared with saline control group.
system may play a very important role in cardiovascular diseases such as hypertension, cardiac hypertrophy, and heart failure.

One question raised in this study centers on the cellular mechanism(s) underlying apelin-induced stimulation of SNA. The effect of apelin on neuronal activity in the RVLM is still not clear. However, a recent study indicates that apelin-13 stimulates magnocellular neurons in the hypothalamic supraoptic nuclei.36 This direct stimulatory effect of apelin on SNA has been confirmed in the RVLM. The effect of apelin on neuronal activity in the RVLM is still not clear. However, a recent study indicates that apelin-13 stimulation of SNA is supported by our preliminary study demonstrating that superfusion of neurons cultured from neonatal rat brain stem with apelin-13 resulted in a 2-fold increase in neuronal firing rate.37 The second messenger signaling systems involved in the apelin-APJ stimulatory effect in neurons appear to be dependent on cell type. For example, apelin-13 is negatively coupled to cAMP production, possibly via G, in APJ-transfected Chinese hamster ovary cells,6,38 whereas the response to apelin in the neuronal cell line 293 is mediated by the elevation of intracellular calcium.39 It has also been reported that apelin stimulates several kinases, such as extracellular signal-regulated kinase 1/2 and AKT.40-42 Thus, additional experiments will be necessary to unravel the signaling pathway involved in the excitatory action of apelin in neurons.

One concern in this study is the identity of the specific apelin peptides that are enhanced in the RVLM of SHR. These apelin peptides are derived from a 77-aa precursor peptide, including preproapelin apelin-36 (42-77), apelin-17 (61-77), and apelin-13 (65-77), and they all share the C-terminal sequence. In the present studies, we used an antibody raised against amino acids 38 to 77 mapping to the C terminus of preproapelin in Western blots, and a real-time RT-PCR probe was designed based on the cDNA coding this area. Thus, we can speculate that the preproapelin levels are enhanced and lead to increases in other apelin peptides, including apelin-13 and apelin-12. This conclusion is supported by another experiment provided in the online data supplement (available at http://circres.ahajournals.org), which shows the enhanced expression of apelin in the RVLM of SHR versus WKY rat using an apelin-12 enzyme immunoassay (Figure I in the online data supplement). Thus, this study raises another question as to which apelin peptide is responsible for the BP elevation observed in the overexpression experiments. Recent studies indicate that the shorter apelin isoforms exhibit greater binding affinity and biological potency than the full-length peptide. Apelin-13 possesses a pyroglutamate substitution at the N terminus, a common posttranslational modification that preserves biological activity by rendering the peptide more resistant to enzymatic cleavage. Thus, the pyroglutamated form of apelin-13 may be the most potent active biological ligand.5,29,46 This hypothesis is supported by the present observations that microinjection of apelin-13 into the RVLM significantly lowers BP in SHR (Online Figure III). In addition, microinjection of this peptide into the RVLM induces increases in BP and HR, which are associated with stimulation of peripheral SNA. Taken together, these results suggest that the apelin system in the RVLM may play a very important role in central BP regulation and the pathogenesis of hypertension.

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Disclosures
None.

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**Expanded Materials and Methods**

**AAV2-mediated transduction of apelin in the RVLM:**

**Viral vector constructs:** AAV2-Apelin and AAV2-GFP were constructed as shown in Figure S2. These constructs contained expression cassettes flanked by the rAAA2 terminal repeats. Expression of enhanced green fluorescent protein (eGFP) and preproapelin were driven by a chicken β-actin promoter (CBA) with a human cytomegalovirus (CMV) enhancer. The full long coding sequence of rat preproapelin (GenBank AF179679, from 301 to 534) was cloned into an AAV2 vector by using a TA cloning kits (Invitrogen, Carlsbad, CA, USA) with the following primers:

**Forward:** 5’-TTA CTA GTG ACC GAG TTG CAG CAT GAA-3’

**Reverse:** 5’-GCA TGC ATT CCT GCT TTA GAA AGG CAT GG-3’

A woodchuck post-transcriptional regulatory element (WPRE), which enhanced the expression of transgenes, was placed at downstream of eGFP and preproapelin. Human embryonic kidney (HEK) 293 cells were used for the virus packaging and vector doses were expressed as genome copies (gc), which is determined with real-time PCR (1).

**RVLM Microinjection:** AAV2-Apelin and AAV2-GFP were injected into the RVLM of WKY rats anesthetized with isoflurane. The blood pressure and heart rate were consistently monitored before and after injection using radiotelemetry as described in the Methods. The rats received AAV2-Apelin, or AAV2-GFP and were sacrificed at the times indicated in Online Figure II. The brains were removed and immediately frozen on dry ice, blocked in the coronal plane, and sectioned at 100 µm thickness in a cryostat (Leica 1950, Nussioch, Germany). The rostral ventrolateral medulla (RVLM) and caudal
ventrolateral medulla (CVLM) was punched and the brain tissue was used for the measurement of apelin mRNA levels using real time RT-PCR and apelin protein levels using ELISA.

**Real-Time RT-PCR**: To measure apelin mRNA levels in the RVLM and CVLM, total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA, USA). Apelin mRNA was analyzed using quantitative real-time RT-PCR as detailed by us previously (2). TaqMan probes specific for rat preproapelin were obtained from Applied Biosystems, Inc. (Foster City, CA). Real time RT-PCR was performed in an Applied Biosystems PRISM 7000 sequence detection system according to the protocol from the manufacturer. Data were normalized to 18S RNA.

**ELISA**: To measure the apelin protein levels in the RVLM and CVLM, the brain tissue was homogenized in a radioimmunoprecipitation assay buffer containing protease inhibitors and centrifuged at 15,000g. The supernatant from homogenates was used to analyze the apelin protein levels with an enzyme immunoassay kit according to the instructions provided (Phoenix Pharmaceuticals, Belmont, CA). This kit is designed to detect apelin-12 and crossly reacts to apelin-13 and apelin-36. The data were expressed as picogram apelin per microgram total protein measured in the same sample with a protein assay kit (Bio Rad Laboratories, Hercules, CA).

**BP recording and microinjection of apelin-neutralizing antibody into the RVLM.**

SHR and WKY rats were anesthetized with inhaled isoflurane as described in Methods. A polyethylene cannula was inserted into the femoral vein for fluid supplementation. BP and HR were measured via a femoral artery cannula connected to a pressure transducer and a Bridge Amplifier (AD Instrument, Colorado Springs, CO). The data were recorded and analyzed with PowerLab software (AD Instrument, Colorado Springs, CO). To investigate the effect of endogenous apelin in the RVLM on blood pressure and heart rate, five SHR and six WKY rats received microinjections of apelin-
neutralizing antibody (50 nl) or PBS vehicle control (50 nl), and changes in BP and HR were measured before and after RVLM microinjection. The RVLM microinjections were performed as described in the Methods and the location of the needle tip of the multiple-barrel glass injection pipette was confirmed by microinjection of L-glutamate.

Expanded Results

**Apelin protein levels are enhanced in the RVLM of SHR versus WKY rats.** In order to detect whether short isoform apelin levels in the RVLM of SHR are also enhanced, we examined apelin protein levels in brain tissue micropunched from the RVLM of SHR and WKY rats using an enzyme immunoassay kit, which is designed to identify apelin-12 and other apelin isoforms. The data are shown in Figure S1, demonstrating that the apelin protein levels, including apelin-12, are enhanced in the RVLM of SHR as compared with WKY rats.

**AAV2 vector-mediated overexpression of apelin in the RVLM.** The viral vectors were constructed as shown in Online Figure II, panel A. To confirm that overexpression of apelin was localized within the RVLM, we examined the apelin mRNA levels in the RVLM and in its adjacent brain cardiovascular regulatory area, CVLM, in WKY rats at the time points shown in Online Figure II B after receiving microinjection of AAV2-Apelin or AAV2-GFP. The results are shown in Online Figure II B, showing that AAV2-Apelin injected into the RVLM increased apelin expression in the RVLM and not in the CVLM. Combined with the results in our immunohistochemistry study in Figure 2, it is demonstrated that the AAV2-Apelin-mediated apelin overexpression was localized within the RVLM. In addition, we also detected the apelin protein levels in the RVLM and in the CVLM of WKY rats after receiving
microinjection of AAV2-Apelin RVLM microinjection using an enzyme immunoassay kit, which is designed to detect apelin-12. The results are shown in Online Figure II C, providing additional evidence that AAV2-Apelin-mediated apelin overexpression is localized within the RVLM in the present study and also that microinjection of AAV2-Apelin into the RVLM increased the expression of apelin peptides, including apelin-12.

Effect of an apelin-neutralizing antibody microinjected into the RVLM on the blood pressure in SHR and WKY rats. To detect the action of endogenous apelin in the RVLM on the blood pressure control, we measured blood pressure before and after microinjection of a specific apelin antibody into the RVLM of SHR and WKY rats. The apelin antibody-induced changes in mean arterial pressure and heart rate are shown in Online Figure III. The baseline and the peak response to apelin antibody injection on blood pressure and heart rate of SHR and WKY rats are listed in the Online Table I. These observations demonstrate that microinjection of apelin antibody into the RVLM significantly decreased mean arterial pressure and heart rate in SHR. However, apelin antibody injected into the RVLM had no significant effect on either blood pressure or heart rate in WKY rats. These results suggest that increased apelin expression in the RVLM may contribute to hypertension in the SHR.

References


Extended Figures and Legends

**Online Figure I:** Apelin protein levels in the RVLM of SHR versus WKY rats. The apelin protein levels in brain tissue micropunches from the RVLM of SHR and WKY rats were determined using an enzyme immunoassay kit, which is designed to detect apelin-12 peptide. Data are means ± SE from five rats in each strain. *P < 0.05 as compared to the WKY rats.

**Online Figure II:** AAV2-GFP and AAV2-Apelin mediated transduction of GFP and apelin into the RVLM of WKY rats. **Panel A:** AAV2-based vectors are used in the present studies. EGFP, enhanced green fluorescent protein; CBA, Chicken β-actin promoter; ITR, inverted terminal repeat; WPRE, woodchuck hepatitis virus post transcriptional control element; bGH, bovine growth hormone. **Panel B:** Real time RT-PCR data showing the expression of apelin mRNA levels in the RVLM and in the CVLM of WKY rats at the times indicated in the figure, after receiving RVLM microinjection of AAV2-Apelin (1 x 10^9 gc in 50 nl) or AAV2-GFP (indicated by the arrow, 1 x 10^9 gc in 50 nl). Data are means ± SE from four rats in each group. *P < 0.01 in comparison to the endogenous apelin mRNA levels in the RVLM and CVLM, respectively. **Panel C:** ELISA data showing apelin protein levels in the RVLM and CVLM of WKY rats at the times indicated in the figure, after receiving RVLM microinjection of AAV2-Apelin (1 x 10^9 gc in 50 nl) or AAV2-GFP (indicated by the arrow, 1 x 10^9 gc in 50 nl). Data are means ± SE from four rats in each group. *P < 0.05 in comparison to the endogenous apelin protein levels in the RVLM and CVLM respectively.
**Online Figure III**: Effect of microinjection of apelin-neutralizing antibody into the RVLM of SHR and WKY rats on blood pressure and heart rate. **Panel A**: A representative arterial pressure recording from SHR before and after receiving RVLM microinjection of apelin antibody (200 µg/ml, 50 nl) or control (rabbit IgG, 200µg/ml, 50 nl). **Panel B**: A bar graph showing the mean arterial pressure (MAP) peak response to the microinjection of apelin antibody (50 nl) into the RVLM of SHR versus WKY rats. Data are means ± SE from six SHR and five WKY rats in each group. *P < 0.01 vs. the MAP changes induced by control injection in SHR or WKY rats respectively. #P<0.05 vs. the MAP changes induced by apelin antibody microinjection in WKY rats. **Panel C**: The heart rate (HR) peak response to microinjection of apelin antibody (50 nl) into the RVLM of SHR versus WKY rats. Data are means ± SE from six SHR and five WKY rats in each group. *P < 0.01 vs. the HR changes induced by control microinjection in SHR or WKY rats respectively. #P<0.05 vs. the HR changes induced by apelin antibody microinjection in WKY rats.

**Online Table I**: Effect of microinjection of apelin-neutralizing antibody into the RVLM on blood pressure in SHR and WKY rats. SAP: systolic arterial pressure; DAP: diastolic arterial pressure; MAP: mean arterial pressure; PP: pulse pressure; HR: heart rate. Values are mean ± SE. *P < 0.01 in comparison to baseline values before injection in the same group rats (n = 5-6 rats/group).
Online Figure II

A

AAV2-Apelin

ITR

CBA

Preproapelin

WPRE

bGH

AAV2-GFP

ITR

CBA

EGFP

WPRE

bGH

B

Apelin mRNA/18s rRNA (Arbitrary Units)

RVLM

CVLM

0 1 2 4 6 8 10 14

The day post gene transfer

C

Apelin (pg/µg total protein)

RVLM

CVLM

0 0.5 1.0 1.5 2.0 2.5

The day post gene transfer
Online Figure III

A

Injection

AP (mmHg)

100
150
200

3 min

100
150
200

B

SHR

WKY

MAP (mmHg)

0
-10
-20
-30
-40

Control

Antibody

* #

C

SHR

WKY

HR (bpm)

0
-10
-20
-30
-40
-50
-60

Control

Antibody

* #
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