Published in final edited form as:

Clin Exp Hypertens. 2016 ; 38(2): 209–217. doi:10.3109/10641963.2015.1081229.

Attenuation of angiotensin type 2 receptor function in the rostral ventrolateral medullary pressor area of the spontaneously hypertensive rat

Tetsuya Kawabe, Masamitsu Iwasa, Kazumi Kawabe, and Hreday N. Sapru

Department of Neurological Surgery, Rutgers New Jersey Medical School, Newark, NJ, USA

Abstract

We hypothesized that blockade of angiotensin II type 2 receptors (AT₂Rs) in the rostral ventrolateral medullary pressor area (RVLM) may elicit sympathoexcitatory responses which are smaller in hypertensive rats compared to normotensive rats. This hypothesis was tested in urethane-anesthetized, artificially ventilated male 14-week-old spontaneously hypertensive rats (SHR). Age-matched male Wistar-Kyoto rats (WKY) and Wistar rats were used as controls. PD123319 (AT₂R antagonist) was microinjected into the RVLM and mean arterial pressure (MAP), heart rate (HR) and greater splanchnic nerve activity (GSNA) were recorded. Increases in MAP, HR and GSNA elicited by unilateral microinjections of PD123319 into the RVLM were significantly smaller in SHR when compared to those in WKY and Wistar rats. Unilateral microinjections of L-glutamate (L-Glu) into the RVLM elicited greater increases in MAP and GSNA in SHR compared to those in WKY. AT₂R immunoreactivity was demonstrated in the RVLM neurons which were retrogradely labeled from the intermediolateral cell column (IML) of the spinal cord. These results indicate that AT₂Rs are present on the RVLM neurons projecting to the IML and their blockade results in sympathoexcitatory responses. Activation of AT₂Rs has an inhibitory influence in the RVLM and these receptors are tonically active. Attenuation of the function of AT₂Rs in the RVLM may play a role in genesis and/or maintenance of hypertension in SHR.

Keywords

Blood pressure; hypertension; microinjections; PD123319; sympathetic nerve activity

Introduction

Angiotensin II (ANG II) is an active peptide of renin-angiotensin system. It is now well established that ANG II plays an important role in cardiovascular regulation within the central nervous system, vasculature and kidney (1–5). All of the components involved in the synthesis of endogenous ANG II are present in the brain (6). Two major types of receptors,

Corresponding author: H.N. Sapru, Ph.D., Department of Neurological Surgery, MSB H-586, Rutgers New Jersey Medical School, 185 South Orange Ave, Newark, N.J. 07103, USA, Telephone: 973-972-5876, FAX: 973-972-5986, sapru@njms.rutgers.edu.

Declaration of interest

The authors report no conflicts of interest.

ANG II type 1 receptors (AT₁Rs) and type 2 receptors (AT₂Rs), mediate the actions of ANG II. Existence of a third type of ANG II receptor has also been reported (3). Neurons and fibers, which are immunoreactive for ANG II as well as AT₁Rs, have been identified in the general area encompassing the rostral ventrolateral medullary pressor area (RVLM) (6–8). Most of the biological actions of ANG II are mediated via AT₁Rs (3–5). In recent years, AT₂Rs have also been implicated in some actions of ANG II (5–6, 9–10). Although the presence of AT₂Rs predominates in fetal life (12), these receptors are present in adults also (2, 11). High levels of AT₂R expression are present in the medulla oblongata (13). Gao et al. (11) have demonstrated the presence of AT₂R protein in the RVLM of the rat.

The sympathoexcitatory neurons located in the RVLM are known to send monosynaptic projections to the intermediolateral cell column (IML) of the thoracolumbar spinal cord (14). It is well established the RVLM plays a critical role in the central regulation of cardiovascular function and the mediation of the baroreceptor, chemoreceptor and cardiopulmonary reflex responses (15–19).

The spontaneously hypertensive rat (SHR) is a commonly used model for studying various aspects of hypertension. It has been reported that microinjections of ANG II into the RVLM of SHR elicited greater increases in mean arterial pressure (MAP) and heart rate (HR) than the Wistar-Kyoto rat (WKY) (20–21). Blockade of AT₁Rs in the RVLM has been shown to elicit depressor responses in SHR (20). Furthermore, in normotensive rats, blockade of AT₁Rs in the RVLM also elicited decreases in MAP after blockade of gamma-aminobutyric acid (GABA) receptors in the same sites (22). These findings indicate that ANG II may be involved in tonic activity of RVLM neurons (23). Recent reports indicate that activation of AT₂Rs may counteract the actions of the AT₁R on cell proliferation, apoptosis and vasomotor action (4–5, 9).

Based on the information presented in the preceding paragraphs, regarding the presence of AT₂Rs in the RVLM, opposing actions of AT₂Rs against AT₁Rs and the importance of ANG II in the RVLM in the pathogenesis of hypertension (24), we hypothesized that blockade of AT₂Rs in the RVLM may elicit pressor responses which would be smaller in hypertensive rats compared to normotensive rats. This hypothesis was tested in this study using Wistar rats, WKY and SHR. There are no reports in the literature regarding the role of AT₂Rs in the RVLM of SHR in cardiovascular regulation.

Methods

General procedures

Fourteen-week old male SHR, and age and sex-matched WKY and Wistar rats (Charles River Laboratories, Wilmington, MA, USA) were used in this study. The animals were housed under controlled conditions with the light and dark cycles set at 12 hour each. Food and water were available to the rats freely. The protocols in this study were approved by the Institutional Animal Care and Use Committee of Rutgers New Jersey Medical School and the experiments were performed according to the NIH guidelines for “The Care and Use of Laboratory Animals, 8th Edition, 2010”. All efforts were made to prevent the suffering of the animals and minimize their discomfort.

The rats were anesthetized initially by administration of isoflurane (2–3% in 100% oxygen) using a nose mask. The trachea was cannulated and artificial ventilation was administered using a rodent ventilator (model 683; Harvard Apparatus, Holliston, MA, USA). The frequency and tidal volume on the ventilator were adjusted to maintain the end tidal CO₂ at 3.5–4.5%. One of the femoral arteries was cannulated for monitoring BP. MAP and HR were derived electronically from BP waves. A femoral vein was cannulated and urethane (1.2–1.4 g/kg) was injected intravenously in 8–9 aliquots at 2-min intervals; the total volume of the anesthetic solution injected into the animal over a period of 16–18 min was 0.4–0.45 ml. Inhalation of isoflurane was stopped as soon as urethane administration was completed. Adequate level of anesthesia was indicated by the absence of a BP response and/or withdrawal of the limb in response to pinching of a hind paw. The rectal temperature was maintained at $37 \pm 0.5^{\circ}\text{C}$ using a temperature controller (model TCAT-2AC, Physitemp Instruments, Clifton, NJ, USA). All of the recordings were stored on a computer hard drive using a data acquisition system obtained from Cambridge Electronic Design Ltd (CED; Cambridge, UK). At the conclusion of the experiment, a high dose of pentobarbital (150 mg/kg, i.v.) was injected into the rats to euthanize them and an incision was made in one of the intercostal muscles to produce pneumothorax; cessation of heart beat indicated that euthanasia was complete. Details of the procedures used in this study were published by us previously (25).

Microinjection technique

The anesthetized rats were placed in a prone position in a stereotaxic instrument (David Kopf Instruments, Tjunga, CA, USA) and the bite bar was adjusted at a level 18 mm below the interaural line. The dorsal medulla was then exposed. Multi-barreled glass-micropipettes (tip size 20–40 μm) were used for microinjections. Each barrel was connected to a picospritzer (General Valve, Fairfield, NJ, USA). The micropipette was introduced into the RVLM perpendicularly; the coordinates for microinjections into the RVLM were: 1.9–2.4 mm rostral to the calamus scriptorius (CS), 2.0–2.3 mm lateral to the midline, and 3.1–3.3 mm deep from the dorsal medullary surface. Microinjections of L-glutamate (L-Glu; 5 mM) were always used to identify the RVLM sites. The duration of microinjection was 10 sec. Controls for microinjections consisted of artificial cerebrospinal fluid (aCSF, pH 7.4). Microinjections into the RVLM were unilateral. The volume of all microinjections into the RVLM was 100 nl. Details of this technique have been reported in one of our earlier publications (26).

Greater splanchnic nerve recording

The greater splanchnic nerve (GSN) was exposed and sectioned at its junction with the celiac ganglion, a small segment was desheathed and its activity was recorded using a bipolar silver wire hook electrode. The activity of whole GSN (GSNA) was amplified ($\times 10,000$ – $20,000$), filtered (100–5000 Hz), digitized and stored on a computer hard drive. The digitized signals were full-wave rectified and integrated over consecutive 1 sec intervals using Spike 2 program (CED, UK). At the end of the experiment, the nerve was sectioned centrally and the remaining activity was considered to be the noise level which was subtracted from the GSNA amplitude. The details of recording from the GSN are mentioned elsewhere (27).

Immunohistochemistry

These experiments were conducted under aseptic conditions. Pentobarbital (50 mg/kg, i.p.) was used to anesthetize Wistar rats. The rats were then placed in a stereotaxic instrument equipped with a rat spinal unit attachment (model 980, David Kopf Instruments) and the dorsal surface of the spinal cord from eighth cervical to third thoracic level was exposed (28–29). Microinjections of fluorogold (FG; 4%, 15 nl) into the IML were made at T1–T2 level (coordinates: 0.8–1 mm lateral to the midline and 0.7–0.9 mm deep from the dorsal surface of the spinal cord) using single-barreled glass micropipettes (tip size 20–40 μ m). After the microinjection was completed, a piece of absorbable gelatin sponge (Surgifoam, Ethicon Inc., Somerville, NJ, USA) was placed on the exposed spinal cord surface and the muscles and skin over the wound were sutured. The rats were then allowed to recover from the anesthesia and kept alive for 7 days. An antibiotic (cefazolin, 30 mg/kg) was administered subcutaneously twice a day for 3 days and one dose of a slow release dosage form of an analgesic (buprenorphine SR, 1 mg/kg) was administered subcutaneously. The animals were then deeply anesthetized with pentobarbital (80 mg/kg, i.p.) and perfused first with heparinized normal saline which was followed by 2% paraformaldehyde solution. The brains were removed and placed in 2% paraformaldehyde for 48 hrs. On completion of the fixation procedure, one side of the brain surface was marked by a shallow cut and serial sections of the medulla and spinal cord were cut (40 μ m) in a vibratome (1000 Plus Sectioning System, The Vibratome Company, St. Louis, MO, USA). The microinjection site of FG (Amax = 361 nm, Emax = 536 nm) in the IML and the retrogradely-labeled cells in the RVLM were visualized under a microscope (model AX70, Olympus Provis, Middlebush, NJ, USA). The sections were photographed (Neurolucida software, version 7.5, MicroBrightField Inc., Williston, VT, USA) and compared with a standard atlas (30). The same sections containing the RVLM were then used for immunostaining of AT₂Rs. The sections were rinsed (rinsing was always done 3 times, 10 min each) with 0.1 M phosphate buffered saline (PBS) and blocked for 60 min at room temperature with 10% normal goat serum (NGS) in 0.1 M PBS containing 0.3% Triton X-100 (TPBS). The sections were incubated for 24 hours at 4°C with rabbit anti-AT₂R antibody (1:500; Abcam, Cambridge, MA, USA; diluted with TPBS containing 3% NGS). After rinsing with PBS, the sections were incubated for 2 hours at 4°C with Cy3-goat anti-rabbit IgG (1:200, Amax = 550 nm, Emax = 570 nm, Jackson Immuno-Research Laboratories Inc., West Grove, PA, USA; diluted with PBS containing 3% NGS). After the completion of incubation with the primary and secondary antibodies in each of these procedures, the sections were rinsed in PBS, mounted on subbed slides, covered with Citifluor mountant medium (Ted Pella Inc., Redding, CA, USA) and coverslipped. The images of the sections were captured (1 μ m apart) using a laser scanning confocal microscope (AIR confocal microscope, Nikon Instruments Inc., Melville, NY, USA). In studies using immunohistochemistry, the specificity of the antibody used for staining AT₂Rs was confirmed by using omission controls.

Histological identification of microinjection sites

After completing the experiments in which microinjections were made into the RVLM, diluted green retrobeads IX (1: 50) were microinjected into the microinjection sites to mark them. As described under “Immunohistochemistry”, the animals were then perfused and

fixed with paraformaldehyde and serial sections of the medulla were cut (40 μ m) in a vibratome and mounted on slides. The microinjection sites in the RVLM were identified under a microscope. The sections were photographed and compared with a standard atlas (30).

Drugs, chemicals and their sources

The following drugs and chemicals were used: FG, green retrobeads IX, isoflurane, L-glutamate monosodium, PD123319 (AT₂R antagonist) (31), pentobarbital sodium and urethane. All of the solutions for the microinjections were freshly prepared in aCSF. The concentration of drugs refers to their salts where applicable. The concentrations of L-Glu, and PD123319 used were selected from our earlier studies (32). The vendors for different drugs and chemicals were as follows: FG from Fluorochrome Inc. (Denver, CO, USA), green retrobeads IX from Lumafluor Inc. (Durham, NC, USA), PD123319 from R & D Systems (Ellisville, MO, USA), and isoflurane from Piramal Critical Care (Bethlehem, PA, USA). All other drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Statistical analyses

The means and standard error of the means (S.E.M.) were calculated for maximum changes in MAP, HR and GSNA in response to microinjections of aCSF, L-Glu and PD123319 into the RVLM. One-way analysis of variance (ANOVA) followed by Tukey-Kramer's multiple test was used for determination of concentration-response of microinjections of L-Glu and PD123319 into the RVLM. For analyses of the GSNA, the integrated signals obtained just before the microinjections of aCSF, L-Glu and PD123319 into the RVLM were averaged over a period of 60 sec. When the responses to these treatments were maximal, the integrated signals were averaged over a period of 60–90 sec. The percentage changes in GSNA elicited by these treatments were calculated and compared between different groups of rats by using Student's unpaired *t*-test. In all cases, the differences were considered significant at $P < 0.05$.

Results

Baseline values for MAP in urethane-anesthetized Wistar rats ($n = 20$), WKY ($n = 24$) and SHR ($n = 24$) were 103.3 ± 2.5 , 90.3 ± 2.6 and 147.1 ± 3.8 mmHg, respectively. Baseline values for HR in Wistar rats, WKY and SHR were 362.2 ± 6.7 , 292.6 ± 10.0 and 339.6 ± 9.1 beats/min (bpm), respectively. Thus, baseline MAP of SHR is significantly ($P < 0.01$) greater than that of WKY and Wistar rats. On the other hand, baseline HR of SHR is significantly ($P < 0.01$) greater than that of WKY but not Wistar rats.

Concentration-response of PD123319 in the RVLM

In all strains of rats, RVLM was first identified by microinjections of L-Glu (5 mM). The increases in MAP in SHR (21.9 ± 0.9 mmHg, $n = 19$) were significantly greater ($P < 0.01$) when compared to those in WKY (16.7 ± 0.6 mmHg, $n = 19$) and Wistar rats (17.3 ± 0.6 mmHg, $n = 20$). Increases in HR in SHR, WKY and Wistar rats were 15.6 ± 1.7 , 18.4 ± 2.4 and 20.2 ± 2.4 mmHg, respectively. Thus, tachycardic responses elicited by microinjections

of L-Glu into the RVLM were not significantly different ($P > 0.05$) in these three strains of rats.

Microinjections of PD123319 into the RVLM elicited increases in MAP in all strains of rats. In each strain of rats, increases in MAP elicited by microinjections of 10 and 20 mM concentrations of PD123319 in the RVLM were significantly ($P < 0.01$ – 0.05) greater than those elicited by microinjections of 2.5 and 5 mM concentrations of PD123319. Increases in MAP elicited by microinjections of 10 mM concentrations of PD123319 into the RVLM of SHR were significantly ($P < 0.01$) smaller than those elicited by microinjections of the same concentration of PD123319 in the RVLM of Wistar rats and WKY. Increases in MAP elicited by microinjections of 20 mM concentrations of PD123319 into the RVLM of SHR were also significantly ($P < 0.01$ – 0.05) smaller than those elicited by microinjections of the same concentration of PD123319 in the RVLM of Wistar rats and WKY (Fig. 1A).

HR increases were also elicited by microinjections of PD123319 into the RVLM in all strains of rats. Increases in HR elicited by microinjections of 10 mM concentrations of PD123319 in the RVLM of Wistar rats and WKY were significantly ($P < 0.05$) greater than those elicited by microinjections of 2.5 and 5 mM concentrations of PD123319 in each strain of rats. Increases in HR elicited by microinjections of 20 mM concentrations of PD123319 in the RVLM of Wistar and WKY were significantly ($P < 0.05$) greater than those elicited by microinjections of 2.5 mM concentrations of PD123319 in each strain of rats. Increases in HR elicited by microinjections of 10 mM concentrations of PD123319 into the RVLM of SHR were significantly ($P < 0.01$) smaller than those elicited by microinjections of the same concentration of PD123319 in the RVLM of Wistar rats and WKY. Increases in HR elicited by microinjections of 20 mM concentrations of PD123319 into the RVLM of SHR were also significantly ($P < 0.01$ – 0.05) smaller than those elicited by microinjections of the same concentration of PD123319 in the RVLM of Wistar rats and WKY (Fig. 1B).

In all strains of rats, increases in MAP and HR elicited by microinjections of 10 mM concentration of PD123319 were not significantly different from those elicited by microinjections of 20 mM concentrations of PD123319. Therefore, 10 mM concentration of PD123319 was used in the experiment described below. The onset, peak and duration of the responses elicited by 10 mM concentration of PD123319 in Wistar rats were 5–10 sec, 1–3 min, and 8–12 min, respectively. Microinjections of aCSF (100 nl) into the RVLM elicited no MAP and HR responses in all strains of rats.

Effect of microinjections of PD123319 into the RVLM on sympathetic nerve activity

Typical tracings of the effects of PD123319 (10 mM) on HR, MAP, pulsatile arterial pressure (PAP) and GSNA in WKY are shown in Fig. 2A. A microinjection of L-Glu (5 mM) into the RVLM elicited increases in HR, MAP, PAP and GSNA (Fig. 2A, a). Five min later, a microinjection of aCSF into the RVLM elicited no HR, MAP, PAP and GSNA responses (Fig. 2A, b). After an interval of 5 min, a microinjection of PD123319 (10 mM) into the RVLM elicited increases in HR, MAP, PAP and GSNA (Fig. 2A, c). Typical tracings of the effects of PD123319 (10 mM) on HR, MAP, PAP and GSNA in SHR are shown in Fig. 2B. A microinjection of L-Glu (5 mM) into the RVLM elicited increases in HR, MAP, PAP and GSNA (Fig. 2B, a). The increases in MAP, PAP and GSNA elicited by a

microinjection of L-Glu in SHR were greater than those in WKY. Five min later, a microinjection of aCSF into the RVLM elicited no HR, MAP, PAP and GSNA responses (Fig. 2B, b). After an interval of 5 min, a microinjection of PD123319 (10 mM) into the RVLM elicited increases in HR, MAP, PAP and GSNA (Fig. 2B, c). However, the increases in HR, MAP, PAP and GSNA elicited by a microinjection of PD123319 (10 mM) in SHR were smaller compared to those in WKY.

Group data for increases in GSNA elicited by microinjections of L-Glu (5 mM), aCSF and PD123319 (10 mM) into the RVLM of WKY and SHR are shown in Fig. 3. The increases in GSNA elicited by microinjections of L-Glu in SHR (Fig. 3D) were significantly ($P < 0.05$) greater than those elicited by microinjections of L-Glu in WKY (Fig. 3A). However, the increases in GSNA elicited by microinjections of PD123319 in SHR (Fig. 3F) were significantly ($P < 0.05$) smaller than those elicited by microinjections of PD123319 in WKY (Fig. 3C). In both strains of rats, microinjections of aCSF (100 nl) into the RVLM elicited no significant GSNA responses (Fig. 3B and E).

Identification of AT₂R immunoreactive RVLM cells retrogradely labeled from the IML

Unilateral microinjection of FG into the IML (T2 level; Fig. 4A) resulted in bilateral retrograde labeling of RVLM neurons with ipsilateral preponderance (labeling on one side is shown in Fig. 4B). Magnified boxed area of Fig. 4B shows RVLM neurons retrogradely labeled with FG (Fig. 4D). Two of these neurons (marked by arrows) were immunoreactive for AT₂Rs (Fig. 4E). Merged image in Fig. 4F indicated that the RVLM neuron retrogradely labeled from IML contained AT₂Rs. Fig. 4G shows another retrogradely RVLM neuron in the same boxed area at a different level (8 μ m deeper). This neuron was also immunoreactive for AT₂Rs (Fig. 4H). Again, the merged image indicated that this RVLM neuron contained AT₂Rs (Fig. 4I). Fig. 4C shows lack of immunoreactivity for AT₂Rs when the antibody was omitted from the protocol (omission control).

Histological identification of microinjection sites in the RVLM

A typical RVLM site marked with green retrobeads IX (1: 50) is shown in Figure 5A. Figs. 5B–F represent composite diagrams of RVLM microinjection sites which were located 1.92–2.40 mm rostral to the CS, 2.0–2.3 mm lateral to the midline, and 3.0–3.3 mm deep from the dorsal medullary surface in Wistar rats, WKY and SHR. The distribution of microinjections sites was similar in the three strains of rats.

Discussion

New findings in this study are as follows: 1) increases in MAP, HR and GSNA elicited by unilateral microinjections of an AT₂R antagonist (PD123319) into the RVLM were significantly smaller in SHR when compared to those in WKY and Wistar rats, and 2) AT₂R immunoreactivity was demonstrated in RVLM neurons retrogradely labeled from the IML.

Non-specific effects, such as distortion of brain tissue at the microinjection site, were not responsible for the cardiovascular and sympathetic responses elicited by microinjections of PD123319 into the RVLM because microinjections of aCSF at the same site did not elicit any cardiovascular or sympathetic responses. The volume of all microinjections into the

RVLM was 100 nl. Selection of this volume was based on one of our earlier studies in which 50 nl and 100 nl volumes of an agonist were injected into the RVLM and it was observed that maximum responses were elicited by a 100 nl volume (26).

Recent reports allude to the emerging concept that various functions of AT₂Rs are opposite to those of AT₁Rs (2–5). Stimulation of AT₂Rs results in inhibition of AT₁R-mediated vasoconstriction and cell proliferation (33–35). In anesthetized rats, stimulation of AT₁Rs in the RVLM has been reported to elicit excitatory responses (3, 7, 11). Therefore, activation of AT₂Rs in the RVLM is expected to elicit opposite responses. Blockade of AT₂Rs is also expected to elicit responses opposite to those elicited by AT₁R blockade. Microinjection of an AT₁R antagonist into the RVLM of normotensive rats does not decrease resting tonic sympathetic activity (36). However, microinjection of an AT₁R antagonist into the RVLM of hypertensive rats does decrease MAP and SNA (20, 23, 37). Microinjections of losartan or L-158809 (another AT₁R antagonist) into the RVLM of SHR, after the blockade of GABARs in this nucleus, resulted in decreases in MAP and SNA (22). In transgenic hypertensive rats with over-expression of renin gene in the brain [(TGR9mREN2)27 rats], bilateral microinjection of losartan into the RVLM elicited a decrease in BP (38). Our results are consistent with the notion that blockade of AT₂Rs elicit responses opposite to those of similar manipulations of AT₁Rs. Thus blockade of AT₂Rs by PD123319 in the RVLM of Wistar rats, WKY and SHR resulted in an increase in MAP, HR and SNA in our study suggesting that endogenously released angiotensin may be tonically activating AT₂Rs in this nucleus. It should be noted that we used PD123319 to block AT₂Rs in the RVLM; although this compound has been reported to be a specific antagonist for AT₂Rs (31), it is desirable to confirm our results by using an additional specific AT₂R antagonist when it becomes available. These responses also suggest that AT₂Rs are tonically active in the RVLM. Sympathoexcitatory effects of PD123319 suggest that RVLM neurons are excited after AT₂R blockade in the RVLM. Indeed, in whole-cell patch-clamp studies on brainstem-spinal cord preparations of AT₁R knockout mice, blockade of AT₂Rs by superfusion of PD123319 elicited depolarization of RVLM neurons (39). The increases in MAP, HR and GSNA elicited by the blockade of AT₂Rs in the RVLM are expected to be mediated via activation of sympathetic preganglionic neurons located in the IML of the spinal cord. Supporting this contention is our observation that RVLM neurons retrogradely labeled by microinjections of a tracer in the IML were immunoreactive for AT₂Rs. As mentioned in the Methods section, the specificity of the antibody used for staining AT₂Rs was confirmed by using omission controls.

We have shown in this study that blockade of AT₂Rs in the RVLM results in increases in MAP, HR and GSNA. These responses after AT₂R blockade may be mediated by activation of AT₁Rs. Consistent with this notion is the report that blockade of AT₂Rs in the RVLM exaggerated increases in MAP and SNA elicited by microinjections of ANG II into the same site in normotensive rats (11).

Activation of AT₂Rs in the RVLM normally causes a decrease in MAP, HR and GSNA. Thus, activation of AT₂Rs in the RVLM has an inhibitory effect on sympathetic drive. Electrophysiological evidence explains the inhibitory effect of AT₂R activation on sympathetic drive. Activation of AT₂Rs has been reported to decrease neuronal activity (3).

Thus, superfusion of ANG II in brainstem-spinal cord preparations of AT₁R knockout mice has been reported to elicit hyperpolarization of RVLM neurons (39). Hyperpolarization of neurons by AT₂R activation is mediated via an increase in delayed rectifier K⁺ currents as demonstrated by whole cell voltage clamp procedures in cocultured neonatal rat hypothalamic and brainstem neurons (40). AT₂R-dependent increase in K⁺ current involves an inhibitory G protein, phospholipase A2 and protein phosphatase 2A (3, 41, 42). Hyperpolarization of RVLM neurons and decrease in their activity induced by AT₂R activation is expected to elicit decreases in MAP, HR and GSNA.

The increases in MAP, HR and GSNA elicited by microinjections of PD123319 into the RVLM in SHR were smaller than those in WKY. It is possible that protein expression for AT₂Rs is decreased and that for AT₁Rs is increased in the RVLM of SHR. Such imbalance in AT₂Rs and AT₁Rs has been reported in the RVLM of rats with stress-induced hypertension (10). The reduction in AT₂R expression in the RVLM of SHR is expected to result in attenuated responses to their blockade. Attenuation of AT₂R function in the RVLM of SHR may also explain exaggerated cardiovascular responses to microinjections of ANG II into the RVLM in these rats (43). Downregulation of AT₂Rs in the RVLM has also been implicated in sympathoexcitation in rats with chronic heart failure (11).

In our study, the increases in MAP elicited by microinjections of L-Glu into the RVLM were greater in SHR than those in WKY and Wistar rats. This observation is in agreement with another report in which exaggerated pressor responses to microinjections of L-Glu into the RVLM of SHR were observed (21). We observed that increases in GSNA elicited by microinjections of L-Glu into the RVLM were greater in SHR than those in WKY and Wistar rats. Considering that the reactivity of resistance vessels in SHR may be altered in SHR (44), we thought it is important to record changes in sympathetic outflow in these experiments. The mechanism of exaggerated pressor and sympathetic responses to microinjections of L-Glu into the RVLM of SHR can be explained as follows. Baroreflex is one of the established regulatory mechanisms of BP control and includes a GABAergic mechanism that tonically inhibits the presympathetic RVLM neurons (15–19, 45). In SHR, baroreflex function is attenuated (46) which results in attenuation of GABAergic input to the presympathetic RVLM neurons and the excitability of presympathetic RVLM neurons increases following this attenuation. Therefore, greater MAP and GSNA responses are elicited by microinjections of L-Glu into the RVLM in SHR compared to the normotensive rats. Moreover, central sympathetic drive has been reported to be increased in hypertension (47). Using whole-cell patch-clamp technique, the firing rate of RVLM neurons has been reported to be faster than that of WKY in brainstem-spinal cord preparations with preserved neuronal network (48). Therefore, exaggerated pressor and sympathetic responses are expected when L-Glu is microinjected into the RVLM of SHR. It is noteworthy that increases in HR elicited by microinjections of L-Glu into the RVLM in SHR were not significantly different from those in normotensive rats. For comparison of HR responses in SHR and normotensive rats, it is important to stimulate nucleus ambiguus by microinjections of L-Glu because vagal input to the heart is relatively more dominant in regulating HR (49).

Conclusion

There is increasing evidence supporting the notion that cross-talk between the AT₁Rs and AT₂Rs may play an important role in the maintenance of cardiovascular homeostasis (5). Ample evidence has been accumulated to show that stimulation of AT₁Rs and AT₂Rs elicit opposite responses (2). A decrease in nocturnal arterial pressure was observed in normal rats with overexpression of AT₂R in the RVLM (50). The results in the present study complement existing literature in which blockade of AT₁Rs and AT₂Rs in the RVLM were demonstrated to elicit opposite responses. Attenuated AT₂R activity in the RVLM observed in our study may play some role in the genesis of hypertension in SHR. Based on these concepts, stimulation of AT₂Rs is considered to be a therapeutic strategy in treating cardiovascular diseases which are associated with sympathoexcitation including hypertension, heart failure and diabetes (3, 4). Activation of AT₂Rs may attenuate the deleterious effects of AT₁R stimulation in such situations. Further studies are needed to elucidate the interaction between AT₁Rs and AT₂Rs in the central nervous system. The results presented in this study provide a platform on which further investigations on this subject can be designed in future.

Acknowledgments

The authors alone are responsible for the content and writing of this article. This research was supported by NIH grants R01-HL024347 and R01-HL076248 awarded to Dr H. N. Sapru.

References

1. Brooks VL. Interactions between angiotensin II and the sympathetic nervous system in the long-term control of arterial pressure. *Clin Exp Pharmacol Physiol*. 1997; 24:83–90. [PubMed: 9043811]
2. Carey RM, Wang ZQ, Siragy HM. Role of the angiotensin type 2 receptor in the regulation of blood pressure and renal function. *Hypertension*. 2000; 35:155–163. [PubMed: 10642292]
3. Gao L, Zucker IH. AT₂ receptor signaling and sympathetic regulation. *Curr Opin Pharmacol*. 2011; 11:124–130. [PubMed: 21159555]
4. Guimond MO, Gallo-Payet N. The Angiotensin II Type 2 receptor in brain functions: An update. *Int J Hypertens*. 2012:1–18. 2012 (ID351758).
5. Schulman IH, Raji L. The angiotensin II type 2 receptor: what is its clinical significance? *Curr Hypertens Rep*. 2008; 10:188–193. [PubMed: 18765088]
6. Allen AM, Moeller I, Jenkins TA, Zhuo J, Aldred GP, Chai SY, Mendelsohn FA. Angiotensin receptors in the nervous system. *Brain Res Bull*. 1998; 47:17–28. [PubMed: 9766385]
7. Head GA, Saigusa T, Mayorov DN. Angiotensin and baroreflex control of the circulation. *Braz J Med Biol Res*. 2002; 35:1047–1059. [PubMed: 12219176]
8. Hu L, Zhu DN, Yu Z, Wang JQ, Sun ZJ, Yao T. Expression of angiotensin II type 1 (AT₁) receptor in the rostral ventrolateral medulla in rats. *J Appl Physiol*. 2002; 92:2153–2161. [PubMed: 11960969]
9. Duke LM, Evans RG, Widdop RE. AT₂ receptors contribute to acute blood pressure-lowering and vasodilator effects of AT₁ receptor antagonism in conscious normotensive but not hypertensive rats. *Am J Physiol Heart Circ Physiol*. 2005; 288:H2289–H2297. [PubMed: 15615839]
10. Du D, Chen J, Liu M, Zhu M, Jing H, Fang J, Shen L, Zhu D, Yu J, Wang J. The effects of angiotensin II and angiotensin-(1–7) in the rostral ventrolateral medulla of rats on stress-induced hypertension. *PLoS One*. 2013; 8:e70976. [PubMed: 23967142]
11. Gao L, Wang WZ, Wang W, Zucker IH. Imbalance of angiotensin type 1 receptor and angiotensin II type 2 receptor in the rostral ventrolateral medulla: potential mechanism for sympathetic overactivity in heart failure. *Hypertension*. 2008; 52:708–714. [PubMed: 18768398]

12. Nuyt AM, Lenkei Z, Palkovits M, Corvol P, Llorens-Cortés C. Ontogeny of angiotensin II type 2 receptor mRNA expression in fetal and neonatal rat brain. *J Comp Neurol.* 1999; 407:193–206. [PubMed: 10213091]
13. Lenkei Z, Palkovits M, Corvol P, Llorens-Cortés C. Distribution of angiotensin II type-2 receptor (AT2) mRNA expression in the adult rat brain. *J Comp Neurol.* 1996; 373:322–339. [PubMed: 8889931]
14. Oshima N, Kumagai H, Onimaru H, Kawai A, Pilowsky PM, Iigaya K, Takimoto C, Hayashi K, Saruta T, Itoh H. Monosynaptic excitatory connection from the rostral ventrolateral medulla to sympathetic preganglionic neurons revealed by simultaneous recordings. *Hypertens Res.* 2008; 31:1445–1454. [PubMed: 18957816]
15. Willette RN, Barcas PP, Krieger AJ, Sapru HN. Vasopressor and depressor areas in the rat medulla: identification by L-glutamate microinjections. *Neuropharmacology.* 1983; 22:1071–1079. [PubMed: 6138727]
16. Sapru HN. Glutamate circuits in selected medullo-spinal areas regulating cardiovascular function. *Clin Exp Pharmacol Physiol.* 2002; 29:491–496. [PubMed: 12010197]
17. Pilowsky PM, Goodchild AK. Baroreceptor reflex pathways and neurotransmitters: 10 years on. *J Hypertens.* 2002; 20:1675–1688. [PubMed: 12195099]
18. Schreihofer AM, Guyenet PG. The baroreflex and beyond: control of sympathetic vasomotor tone by GABAergic neurons in the ventrolateral medulla. *Clin Exp Pharmacol Physiol.* 2002; 29:514–21. [PubMed: 12010201]
19. Guyenet PG. The sympathetic control of blood pressure. *Nature Rev Neurosci.* 2006; 7:335–346. [PubMed: 16760914]
20. Ito S, Komatsu K, Tsukamoto K, Kanmatsuse K, Sved AF. Ventrolateral medulla AT1 receptors support blood pressure in hypertensive rats. *Hypertension.* 2002; 40:552–559. [PubMed: 12364362]
21. Lin Y, Matsumura K, Kagiya S, Fukuhara M, Fujii K, Iida M. Chronic administration of olmesartan attenuates the exaggerated pressor response to glutamate in the rostral ventrolateral medulla of SHR. *Brain Res.* 2005; 1058:161–166. [PubMed: 16143317]
22. Tagawa T, Fontes MAP, Potts PD, Allen AM, Dampney RAL. The physiological role of AT1 receptors in the ventrolateral medulla. *Braz J Med Biol Res.* 2000; 33:643–652. [PubMed: 10829092]
23. Dampney RAL, Fontes MAP, Hirooka Y, Horiuchi J, Potts PD, Tagawa T. Role of angiotensin II receptors in the regulation of vasomotor neurons in the ventrolateral medulla. *Clin Exp Pharmacol Physiol.* 2002; 29:467–472. [PubMed: 12010194]
24. Ferrario, CM.; Moriguchi, A.; Broshihan, KB. Angiotensin mechanism in hypertension. In: Ganten, D.; de Jong, W., editors. *Handbook of hypertension.* Vol. 16. Amsterdam: Elsevier Science Press; 1994. p. 441–461.
25. Kawabe T, Kawabe K, Sapru HN. Tonic gamma-aminobutyric acid-ergic activity in the hypothalamic arcuate nucleus is attenuated in the spontaneously hypertensive rat. *Hypertension.* 2013; 62:281–287. [PubMed: 23774228]
26. Kawabe T, Chitravanshi VC, Kawabe K, Sapru HN. Cardiovascular effects of adrenocorticotropin microinjections into the rostral ventrolateral medullary pressor area of the rat. *Brain Res.* 2006; 1102:117–126. [PubMed: 16797500]
27. Kawabe T, Chitravanshi VC, Kawabe K, Sapru HN. Cardiovascular function of a glutamatergic projection from the hypothalamic paraventricular nucleus to the nucleus tractus solitarius in the rat. *Neuroscience.* 2008; 153:605–617. [PubMed: 18424005]
28. Kawabe T, Chitravanshi VC, Nakamura T, Kawabe K, Sapru HN. Mechanism of heart rate responses elicited by chemical stimulation of the hypothalamic paraventricular nucleus in the rat. *Brain Res.* 2009; 1248:115–126. [PubMed: 19022229]
29. Sundaram K, Murugaian J, Sapru H. Cardiac responses to the microinjections of excitatory amino acids into the intermediolateral cell column of the rat spinal cord. *Brain Res.* 1989; 482:12–22. [PubMed: 2565136]
30. Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates.* 6th. London: Academic Press; 2007.

31. Blankley CJ, Hodges JC, Klutchko SR, Himmelsbach RJ, Chucholowski A, Connolly CJ, Neergaard SJ, Van Nieuwenhze MS, Sebastian A, Quin J3rd, Essenberg AD, Cohen DM. Synthesis and structure-activity relationships of a novel series of non-peptide angiotensin II receptor binding inhibitors specific for the AT2 subtype. *J Med Chem.* 1991; 34:3248–3260. [PubMed: 1956044]
32. Kawabe T, Kawabe K, Sapru HN. Cardiovascular effect of angiotensin-(1–12) in the caudal ventrolateral medullary depressor area of the rat. *Am J Physiol Heart Circ Physiol.* 2014; 306:H438–H449. [PubMed: 24285114]
33. Hannan RE, Davis EA, Widdop RE. Functional role of angiotensin II AT2 receptor in modulation of AT1 receptor-mediated contraction in rat uterine artery: involvement of bradykinin and nitric oxide. *Br J Pharmacol.* 2003; 140:987–995. [PubMed: 14530222]
34. Kaschina E, Unger T. Angiotensin AT1/AT2 receptors: regulation, signaling and function. *Blood Press.* 2003; 12:70–88. [PubMed: 12797627]
35. Stoll M, Steckelings UM, Paul M, Bottari SP, Metzger R, Unger T. The angiotensin AT2-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J Clin Invest.* 1995; 95:651–657. [PubMed: 7860748]
36. Hirooka Y, Potts PD, Dampney RAL. Role of angiotensin II receptor subtypes in mediating the sympathoexcitatory effects of exogenous and endogenous angiotensin peptides in the rostral ventrolateral medulla of the rabbit. *Brain Res.* 1997; 772:107–114. [PubMed: 9406962]
37. Allen AM. Blockade of angiotensin AT1-receptors in the rostral ventrolateral medulla of spontaneously hypertensive rats reduces blood pressure and sympathetic nerve discharge. *J Renin-Angiotensin-Aldosterone Syst.* 2001; 2(suppl 1):S120–S124.
38. Fontes MA, Baltatu O, Caligiore SM, Campagnole-Santos MJ, Ganten D, Bader M, Santos RA. Angiotensin peptides acting at rostral ventrolateral medulla contribute to hypertension of TGR(mREN2)27 rats. *Physiol Genomics.* 2000; 2:137–142. [PubMed: 11015592]
39. Matsuura T, Kumagai H, Onimaru H, Kawai A, Iigaya K, Onami T, Sakata K, Oshima N, Sugaya T, Saruta T. Electrophysiological properties of rostral ventrolateral medulla neurons in angiotensin II 1a receptor knockout mice. *Hypertension.* 2005; 46:349–354. [PubMed: 15998710]
40. Kang J, Sumners C, Posner P. Angiotensin II type 2 receptor-modulated changes in potassium currents in cultured neurons. *Am J Physiol.* 1993; 265:C607–C616. [PubMed: 8214016]
41. Gelband CH, Zhu M, Lu D, Reagan LP, Fluharty SJ, Posner P, Raizada MK, Sumners C. Functional interactions between neuronal AT1 and AT2 receptors. *Endocrinology.* 1997; 138:2195–2198. [PubMed: 9112419]
42. Kang J, Richards EM, Posner P, Sumners C. Modulation of the delayed rectifier K⁺ current in neurons by an angiotensin II type 2 receptor fragment. *Am J Physiol.* 1995; 268:C278–C282. [PubMed: 7840157]
43. Zhu DN, Moriguchi A, Mikami H, Higaki J, Ogihara T. Central amino acids mediate cardiovascular response to angiotensin II in the rat. *Brain Res Bull.* 1998; 45:189–197. [PubMed: 9443839]
44. Norellson E, Folkow B, Hallback-Nordlander M. Cardiovascular ‘reactivity’ to graded splanchnic nerve stimulation in spontaneously hypertensive and normotensive control rats. *Acta Physiol Scand.* 1979; 106:169–176. [PubMed: 159600]
45. Dampney RA, Horiuchi J, Tagawa T, Fontes MA, Potts PD, Polson JW. Medullary and supramedullary mechanisms regulating sympathetic vasomotor tone. *Acta Physiol Scand.* 2003; 177:209–218. [PubMed: 12608991]
46. Sapru HN, Krieger AJ. Role of receptor elements in baroreceptor resetting. *Am J Physiol.* 1979; 236:H174–H182. [PubMed: 434168]
47. Abboud FM. The sympathetic system in hypertension. State-of-the-art review. *Hypertension.* 1982; 4:208–225. [PubMed: 7040239]
48. Matsuura T, Kumagai H, Kawai A, Onimaru H, Imai M, Oshima N, Sakata K, Saruta T. Rostral ventrolateral medulla neurons of neonatal Wistar-Kyoto and spontaneously hypertensive rats. *Hypertension.* 2002; 40:560–565. [PubMed: 12364363]
49. Mendelowitz D. Advances in parasympathetic control of heart rate and cardiac function. *News Physiol Sci.* 1999; 14:155–161. [PubMed: 11390842]

50. Gao L, Wang W, Wang W, Li H, Sumners C, Zucker IH. Effects of angiotensin type 2 receptor overexpression in the rostral ventrolateral medulla on blood pressure and urine excretion in normal rats. *Hypertension*. 2008; 51:521–527. [PubMed: 18086951]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1.

Concentration-response of PD123319 into the RVLM. Open squares: Wistar rats, open circles: WKY and dark circles: SHR. A: Increases in MAP elicited by microinjections of PD123319 into the RVLM of all strains. Increases in MAP elicited by microinjections of 2.5, 5, 10 and 20 mM concentrations of PD123319 were as follows: 4.8 ± 1.0 (n = 5), 6.8 ± 0.6 (n = 5), 16.0 ± 0.5 (n = 5) and 15.0 ± 1.2 mmHg (n = 5), respectively (Wistar rats), 6.3 ± 1.3 (n = 4), 9.8 ± 1.1 (n = 5), 16.4 ± 0.5 (n = 5) and 16.8 ± 1.1 mmHg (n = 5), respectively (WKY) and 5.0 ± 0.7 (n = 4), 5.8 ± 1.0 (n = 5), 10.0 ± 1.1 (n = 5) and 9.6 ± 0.9 mmHg (n =

5), respectively (SHR). B: Increases in HR elicited by microinjections of PD123319 into the RVLM of all strains. Increases in HR elicited by microinjections of 2.5, 5, 10 and 20 mM concentrations of PD123319 were as follows: 8.8 ± 0.7 , 13.4 ± 1.9 , 23.6 ± 3.3 and 21.0 ± 2.2 bpm, respectively (Wistar rats), 13.0 ± 2.0 , 15.8 ± 3.5 , 25.2 ± 3.9 and 27.4 ± 3.7 bpm, respectively (WKY) and 6.5 ± 2.6 , 9.2 ± 1.9 , 11.6 ± 2.4 and 9.8 ± 2.3 mmHg, respectively (SHR). * $P < 0.05$ compared to responses elicited by 10 mM concentration of PD123319 in Wistar rats and WKY. † $P < 0.01$ – 0.05 compared to responses elicited by 20 mM concentration of PD123319 in Wistar rats and WKY. Abbreviations: bpm, beats/min; HR, heart rate; MAP, mean arterial pressure; RVLM, rostral ventrolateral medullary pressor area; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats. In this and other figures, the volume of all microinjections was 100 nl. All values are expressed as the means $\pm$ standard error of the means.

Fig. 2.

Traces showing the effect of blockade of AT₂Rs in the RVLM. In each panel, top trace: HR (bpm), 2nd trace: MAP (mmHg), 3rd trace: PAP (mmHg), and bottom trace: integrated GSNA (μV/sec). A: In WKY, RVLM was first identified by a microinjection of L-Glu (5 mM); increases in HR, MAP, PAP and GSNA were observed (a). Five min later, a microinjection of aCSF into the same site elicited no HR, MAP, PAP and GSNA responses (b). Within 5 min, a microinjection of PD123319 (10 mM) into the same site elicited increases in HR, MAP, PAP and GSNA (c). B: In SHR, a microinjection of L-Glu (5 mM) into the RVLM elicited increases in HR, MAP, PAP and GSNA (a); the increases in MAP, PAP and GSNA were greater than those in WKY. Five min later, a microinjection of aCSF into the same site elicited no responses (b). After an interval of 5 min, a microinjection of PD123319 (10 mM) into the same site elicited increases in HR, MAP, PAP and GSNA which were smaller than those in WKY (c). Abbreviations: aCSF, artificial cerebrospinal fluid; GSNA, greater splanchnic nerve activity; L-Glu, L-glutamate; μV: microvolts; PAP, pulsatile arterial pressure.

Fig. 3.

Group data showing the effect of AT₂R blockade in the RVLM on GSNA. WKY (open bars). SHR (dark bars). In WKY, increases in GSNA elicited by microinjections of L-Glu (5 mM) (A), aCSF (B) and PD123319 (10 mM) (C) into the RVLM were 43.6 ± 4.6 , 0.7 ± 1.7 and 37.4 ± 4.3 %, respectively. In SHR, increases in GSNA elicited by microinjections of L-Glu (5 mM) (D), aCSF (E) and PD123319 (10 mM) (F) into the RVLM were 59.6 ± 4.7 , 0.6 ± 1.2 and 22.9 ± 2.1 %, respectively. *P < 0.05.

Fig. 4. Identification of AT₂R immunoreactive RVLM neurons retrogradely labeled from the IML. A: A microinjection site (arrow) of FG (4%, 15 nl) in the IML (T2 level). B: Ipsilateral RVLM neurons labeled retrogradely with FG. C: Omission of the antibody for AT₂Rs resulted in no staining in the RVLM (omission control). D: Magnified image of the boxed area in B obtained by confocal microscopy shows retrogradely labeled RVLM neurons. E: AT₂R immunoreactive neurons in the section shown in D. F: merged images of D and E show that the retrogradely labeled RVLM neuron contained AT₂Rs (arrows). G: Magnified

image of the boxed area in B obtained at a deeper level (8 μm apart) by confocal microscopy shows another retrogradely labeled RVLM neuron (arrow). H: the neuron shown in G was immunoreactive for AT_2Rs . I: merged images of G and H show that this retrogradely labeled RVLM neuron contained AT_2Rs . FG: fluorogold; IML: the intermediolateral cell column; nAmb: the nucleus ambiguus. Bars: panel A = 1 mm, panel B = 100 μm and panel C-I = 50 μm .

Fig. 5.

Histological identification of microinjection sites in the RVLM.

A: A typical microinjection site in the RVLM marked with green retrobeads IX (100 nL) in SHR (arrow). B-F: composite diagrams of sections at different levels showing microinjection sites in the RVLM. Microinjection sites in the RVLM: Wistar rats (open squares), WKY (open circles) and SHR (dark circles). The numbers mentioned below each section drawing indicate the levels rostral to the calamus scriptorius. In all strains of rats, microinjections were made unilaterally (left or right side). In the diagrams, for

simplification, microinjection sites in Wistar rats and WKY are shown at the right side and those in SHR are shown at left side. Bar in panel A = 1 mm.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript