AT_1 receptor in rostral ventrolateral medulla mediating blunted baroreceptor reflex in spontaneously hypertensive rats[1]

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KEY WORDS angiotensin II; antisense oligodeoxynucleotide; hypertension; baroreceptor reflex; rostral ventrolateral medulla

ABSTRACT

AIM: To determine the role of AT_1 receptor in the rostral ventrolateral medulla (RVLM) in mediating the blunted baroreceptor reflex in spontaneously hypertensive rats (SHR). **METHODS:** Intravenous injections of graded doses of phenylephrine (1, 5, 10, 20, and 40 μg/kg) increased the blood pressure to elicit the baroreceptor reflex in both SHR and normotensive Wistar rats anesthetized with urethane. The baroreceptor reflex sensitivity (BRS) was determined before and after microinjection of Ang II, losartan, or AT_1 receptor antisense oligodeoxynucleotides into the RVLM. AT_1 receptor protein level in the RVLM was measured by Western blotting. **RESULTS:** The BRS was significantly decreased in SHR compared with normal rats. Bilateral microinjection of AT_1 receptor antagonist losartan (250 nmol/h) into the RVLM partly reversed the blunted BRS in SHR, but had no significant effect on the BRS in normal rats. Ang II (1.5 nmol/h) significantly inhibited the BRS in normal rats, which was completely abolished by pretreatment with losartan. However, no significant change in the BRS was observed after microinjection of Ang II into the RVLM in SHR. Bilateral microinjection of AT_1 receptor antisense oligodeoxynucleotides (ASODN) into the RVLM partially recovered the blunted BRS in SHR after 3 h, but no significant change in the BRS was observed in normal rats. The AT_1 receptor protein level significantly decreased after administration of ASODN. **CONCLUSION:** Blockage of AT_1 receptor or inhibition of AT_1 receptor protein synthesis in the RVLM enhanced the BRS in SHR, suggesting that the enhanced activities of AT_1 receptor in the RVLM contribute to the blunted BRS in SHR.

INTRODUCTION

The baroreceptor reflex plays a crucial role in the regulation of cardiovascular activity. Its main function is to buffer blood pressure changes by regulating sympathetic and parasympathetic activity. It has been found that the baroreceptor reflex sensitivity (BRS) was blunted and that the regulation of heart rate and the sympathetic reflex was attenuated in several hypertensive animal models[1;2] as well as essential and secondary hypertensive patients[3]. Administration of angiotensin II (Ang II) into the forth cerebral ventricle in conscious normotensive rabbits was able to diminish the cardiac BRS, whereas blockade of the AT_1 receptors with losartan induces the opposite effect, suggesting that endogenous Ang II in the brain stem elicits a tonic inhibitory effect

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on the cardiac baroreflex[4]. It is well known that an intrinsic angiotensin system exists in the brain. Angiotensinogen, the precursor molecule for angiotensin I, II, III, and angiotensin-converting enzyme (ACE), and aminopeptidases A and N may all be synthesized within the brain. Angiotensin receptors of subtypes AT_1, AT_2, and AT_4 are also plentiful in the brain[5,6]. The rostral ventrolateral medulla (RVLM) is well established as a basic area in central cardiovascular regulation and a major source of sympathetic activation[7,8]. The RVLM is rich in AT_1 receptors[9]. Our previous study found that central Ang II inhibited the BRS in the chronic heart failure rats[10], and recent data indicated that microinfusion of Ang II into the RVLM resulted in a significant inhibition of the BRS, which was almost completely abolished by pretreatment with microinjection of AT_1 receptor antagonist losartan, suggesting that AT_1 receptors played an important role in modulating BRS in the normotensive rats[11]. However, whether the AT_1 receptor in the RVLM contributes to the blunted BRS in hypertension is unknown. The present study evaluated the role of AT_1 receptor in the RVLM for the attenuation of the BRS in the spontaneously hypertensive rats (SHR) by administration of antisense oligodeoxynucleotides against AT_1 receptor mRNA, AT_1 receptor antagonist losartan, and Ang II into the RVLM.

MATERIALS AND METHODS

Chemicals ANG II was purchased from Sigma Co. Losartan was a gift from Merck Co. Antisense oligodeoxynucleotides (ASODN) against AT_1 receptor mRNA were 15-mer oligodeoxynucleotides (5'-TAACTGTGCCTGCCA-3'), and scrambled antisense oligodeoxynucleotides (ScrODN, 5'-CTTACTAGCCTAGGC-3') were used as sequence controls. Both ASDON and ScrODN were all synthesized and purified at Sigma Co. Both AT_1 polyclonal antibody and the secondary antibody were bought from Santa Cruz Biotechnology.

Animals Forty male SHR and forty-eight male normotensive Wistar rats weighing between 300 and 400 g were used. All animals were obtained from Shanghai Laboratory Animal Center, Chinese Academy Sciences. Each rat was anesthetized with intraperitoneal injection of urethane (1.5 g/kg). Supplemental doses of anesthesia were administered at one-tenth initial dose per hour. The adequacy of this anesthetic recipe was ascertained by the lack of dodge reaction to hind paw pinching. Animals were tracheotomized and artificially ventilated.

Arterial pressure and heart rate recording Polyethylene catheters (PE50) filled with heparinized saline were inserted into the right femoral artery and vein for arterial pressure recording and drug administration respectively. Mean arterial pressure (MAP) was monitored by a solid-state strain-gauge transducer and connected to bridge amplifier (ML122, ADInstruments, Australia), and heart rate (HR) was determined by the arterial pressure waves. All variables were recorded and saved to a PowerLab digital acquisition system (PowerLab/8sp ADInstruments, Australia).

RVLM orientation The rat was placed in a stereotaxic instrument (Stoelting; Chicago, IL) with the head in the horizontal position and the skull was exposed through an incision on the midline of the scalp. A cannula (outer diameter 0.6 mm and inner diameter 0.3 mm) was positioned in the RVLM according to the Paxinos and Watson rat atlas[11], which is 12.7 mm posterior, 2.1 mm lateral to the bregma, and 10.1 mm ventral to the zero level. At the end of the experiment, 0.1 μL Evans blue dye (2 %) was injected into the microinjection site. The rat was euthanized with an overdose of anesthesia. The brain was rapidly removed and placed in 10 % formalin. The brain was sectioned and the microinjection site was verified. Only the data of rats whose microinjection sites were within the boundaries of the RVLM were used for analysis.

RVLM administration The rats were randomly divided into five groups in SHR and six groups in normotensive Wistar rats (n=8 for each group). In the first part of the experiment, normal saline, Ang II (1.5 nmol/h) and losartan (250 nmol/h) were microinjected into the RVLM in three groups of SHR and three groups of Wistar rats. Additional group of losartan plus Ang II was used in Wistar rats. Microinjection was performed with a microsyringe pushed by motor-driven micromanipulator (IM-1, Narishige, Japan) at a constant rate of 500 nL/h. In the second part, ASODN (275 pmol/100 nL) and ScrODN (275 pmol/100 nL) were microinjected into the RVLM in two groups of SHR and two groups of Wistar rats. The microinjection was completed within 30 s, and the volume was 100 nL.

Determination of BRS The BRS was evaluated before and after central microinjection by recording HR changes in response to MAP changes produced by repeated bolus intravenous injections of graded doses of phenylephrine (1, 5, 10, 20, and 40 μg/kg). After phe-

nylephrine administration, the blood pressure elevated immediately and reached its maximum in 3 s, and the heart rate decreased to the lowest level in 5 s. The effects of phenylephrine completely recovered to control level within 2 min. Phenylephrine injections were separated with an interval of at least 3 min for a complete recovery. The baroreceptor reflex sensitivity was then estimated in each rat by fitting a least-squares regression line for the relationship between changes in HR and MAP for each data point obtained with graded injection of phenylephrine. The slope of the line expressing the relationship (beats·min^{-1}·mmHg^{-1}) was used as an index of baroreceptor reflex sensitivity.

Western blot The SHR and normotensive Wistar rats were sacrificed with an overdose of pentobarbital 4 h after microinjection of the ASODN or ScrODN into the RVLM (n=3 for each group). The brain was removed and immediately frozen in liquid nitrogen and stored at -70 ºC until sectioned. A 450 μm-thick coronal section was cut through the medulla oblongata and incorporated the RVLM area which was punched out with a 15 gauge needle (ID 1.5 mm). The punched tissue was put in 0.5 mL of TRI Reagent (MRC, Cincinnati, Ohio) and homogenized by sonication. The total proteins in the homogenate were extracted according to the TRI Reagent manufacturers' instructions. AT_1-R protein in the RVLM was measured with Western blot method. The methodology for Western blot was similar to that described by Li *et al* [12]. For Western blot, the primary antibody was rabbit-anti rat AT_1 polyclonal antibody, 1:1000 in 5 mL of 5 milk-TBST solution. The secondary antibody was goat-anti rabbit IgG, peroxidase conjugated, 1:2500 in 5 mL of 5 % milk-TBST solution.

Statistical analysis All data were presented as mean±SD. Comparisons between two observations (before *vs* after administration) in the same animal were assessed by paired t test. Differences among groups were assessed by one-way ANOVA. The criterion for statistical significance was set at P<0.05.

RESULTS

BRS, MAP, and HR Intravenous injection of graded doses of phenylephrine (1, 5, 10, 20, and 40 μg/kg) resulted in a dose-related elevation of MAP and a secondary decrease in HR due to baroreceptor reflex (Fig 1). Although the MAP of SHR was significantly higher than that of Wistar rats (+48.8 %, P<0.01), the BRS of SHR was significantly decreased compared with that of Wistar rats (-60.0 %, P<0.01). However, there was no significant difference in HR between SHR and Wistar rats (Tab 1).

Effect of Ang II and losartan on BRS In SHR, bilateral microinjection of Ang II (1.5 nmol/h) into the RVLM had no significant effect on the BRS. However, AT_1 receptor antagonist losartan (250 nmol/h) partly reversed the blunted BRS (-1.5±0.3 *vs* -2.3±0.5

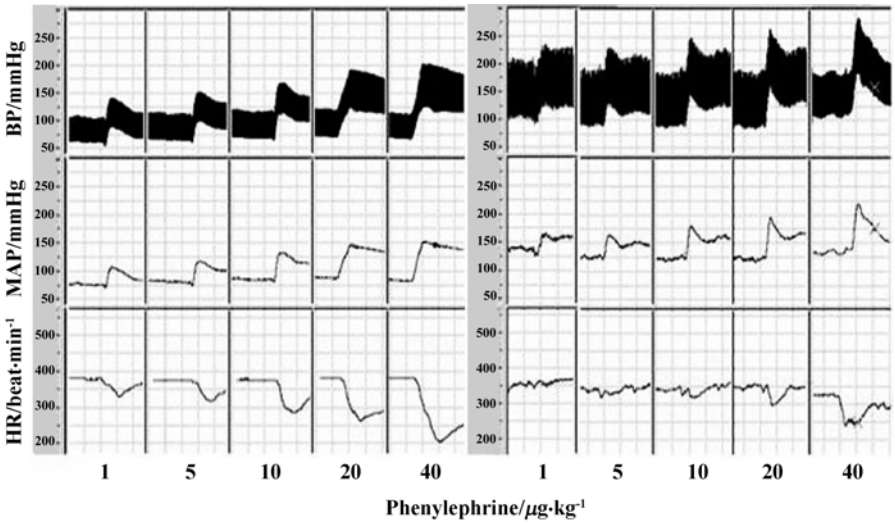

Fig 1. Representative recordings showing the MAP increase and reflex bradycardia produced by repeated bolus intravenous injections of graded doses of phenylephrine (1, 5, 10, 20, and 40 μg/kg) in normotensive Wistar rats (left) and SHR (right). Intravenous injections of phenylephrine in SHR resulted in stronger pressor response but weaker bradycardia response.

Tab 1. The baseline of MAP, HR, and BRS in SHR and normotensive Wistar rats. Mean±SD. $^{c}P<0.01$ *vs* Wistar rats.

	Wistar rats	SHR
n	16	18
MAP/mmHg	91±13	135±18^{c}
HR/bpm	396±18	379±39
BRS/beats·min^{-1}·mmHg^{-1}	-3.5±0.4	-1.4±0.4^{c}

beats·min^{-1}·mmHg^{-1}, $P<0.01$, Fig 2). Conversely, in normotensive rats, BRS was decreased by 40.5 % by the same dose of Ang II (-3.7±0.9 *vs* -2.3±0.5 beats·min^{-1}·mmHg^{-1}, $P<0.05$). Although losartan had no significant effect on the BRS, the effect of microinjection of Ang II to blunt the baroreceptor reflex sensitivity was almost abolished by pretreatment with losartan (Fig 3).

Effect of ASODN on BRS The BRS was determined before and 3 h after bilateral microinjection of ASODN (275 pmol) against AT_1 receptor mRNA or ScrODN into the RVLM. ASODN significantly increased the blunted BRS in the SHR (-1.3±0.2 *vs* -2.2±0.4 beats·min^{-1}·mmHg^{-1}, $P<0.01$), but ASODN had no significant effect on the BRS in normotensive Wistar rats. No effect of ScrODN on the BRS was observed in SHR as well as in Wistar rats (Fig 4).

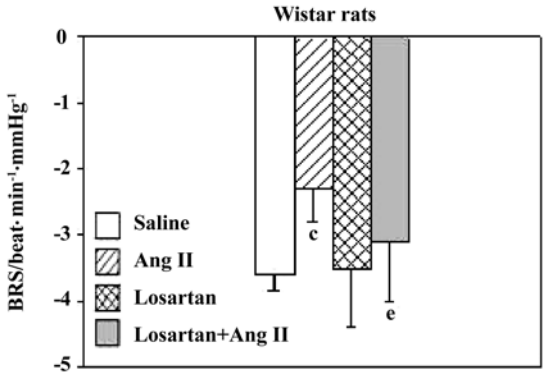

Fig 3. Pretreatment with losartan blocked the effect of Ang II on the BRS in normotensive Wistar rats. *n*=8 for each group. Mean±SD. $^{c}P<0.01$ *vs* saline. $^{e}P<0.05$ *vs* Ang II.

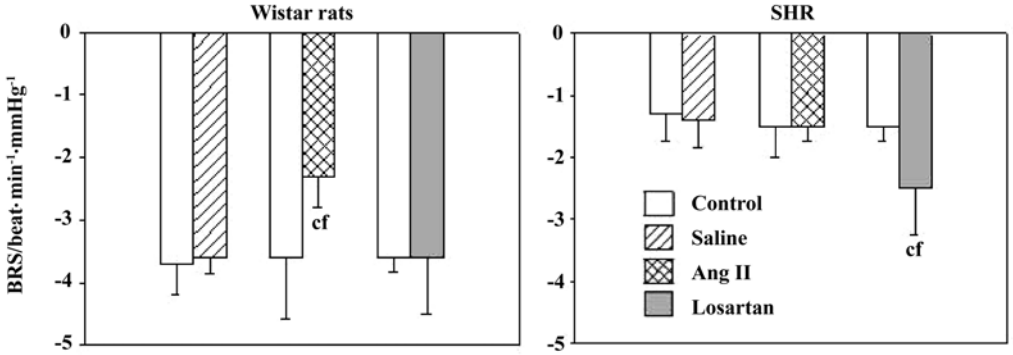

Fig 2. The effect of microinjection of Ang II and losartan into the RVLM on the BRS in SHR and normotensive Wistar rats. *n*=8 for each group. Mean±SD. $^{c}P<0.01$ *vs* control. $^{f}P<0.01$ *vs* saline.

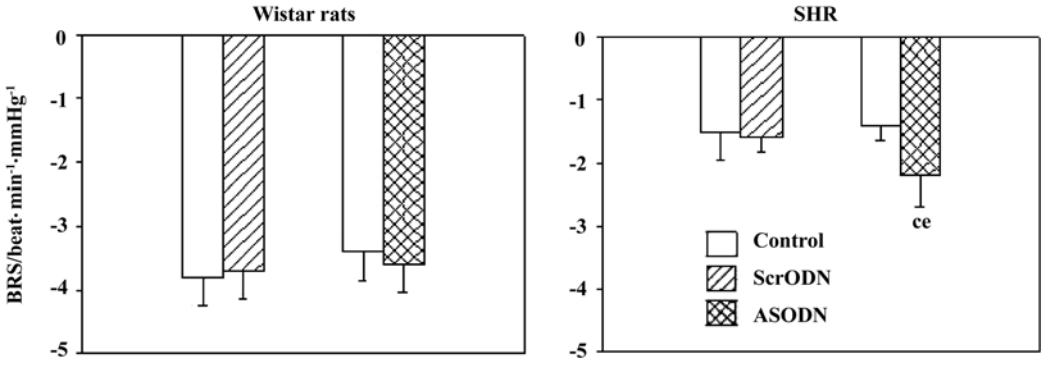

Fig 4. The effect of microinjection of ASODN against AT_1 receptors and control ScrODN on BRS in normotensive Wistar rats and SHR. *n*=8 for each group. Mean±SD. $^{c}P<0.01$ *vs* control. $^{e}P<0.05$ *vs* ScrODN.

Effect of ASODN on the AT_1 receptor protein expression in the RVLM Compared with microinjection of ScrODN into the RVLM, ASODN resulted in a decrease of AT_1 receptor protein expression in the RVLM 4 h after microinjection of ASODN into the RVLM in SHR, but failed to elicit significant change in normotensive Wistar rats (Fig 5).

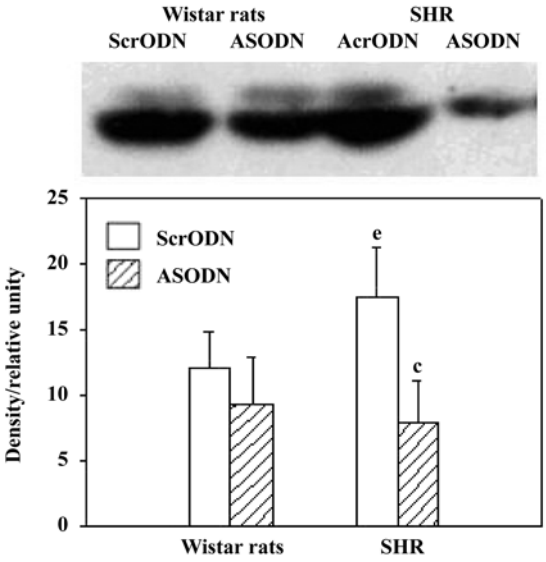

Fig 5. Western blot analysis showed that microinjection of ASODN inhibited the AT_1 receptor protein expression in the RVLM. *n*=3 for each group. Mean±SD. $^cP<0.01$ *vs* ScrODN. $^eP<0.05$ *vs* Wistar rats.

DISCUSSION

The central Ang II plays an important role in modulating sympathetic outflow and baroreflex[10]. Our previous study indicated that AT_1 receptors in the RVLM played an important role in modulating cardiac BRS in the normotensive rats[11]. Whether the AT_1 receptor in the RVLM contributes to the blunted BRS in hypertension is a very interesting question. The primary finding in the present study was that microinjection of AT_1 receptor antagonist losartan or ASODN against AT_1 receptor mRNA into the RVLM partially reversed the blunted BRS in SHR. These results indicated that the enhanced tonic activity of endogenous AT_1 receptor in the RVLM contributed to the blunted BRS in SHR. Our present result is consistent with a recent study showing that the density of the AT_1 receptor in the RVLM is significantly increased in SHR compared with normotensive Wistar rats[13].

Our previous study found that AT_1 receptor protein level was reduced by 38.4 % at 5 h after microinjection of the ASODN against AT_1 receptor mRNA into the paraventricular nucleus[14]. The present study showed a similar decrease of AT_1 receptor protein level in the RVLM 4 h after microinjection of the ASODN into the RVLM. It is known that AT_1 receptor ASODN can selectively inhibit the AT_1 receptor protein expression rather than disturbing other protein expression[15], and result in obvious effect 2-3 h after administration[14,16]. Therefore, the effect of ASODN on the BRS is related to the inhibition of synthesis of AT_1 receptor protein in the RVLM. However, bilateral microinjection of losartan into the RVLM only partially recovered the blunted BRS. Our previous study showed that microinjection of the same dose of losartan into the paraventricular nucleus completely abolished the enhanced cardiac sympathetic afferent reflex[17]. These results suggest that AT_1 receptor in the RVLM is only partly responsible for the inhibition of BRS in SHR.

Antisense oligodeoxynucleotides have been used to determine the role of Ang II in neural control of the cardiovascular system[18]. A special advantage of using antisense techniques is that distinct receptor populations can be targeted. The antisense oligodeoxynucleotide is taken up locally to interfere with synthesis of receptors in cell bodies at the injection site. However, receptors synthesized at remote locations and transported to the site via cells with fiber terminals projecting to the area are spared[16]. Therefore, unlike receptor antagonists that block all receptors in the area of the injection, antisense technique affords a window of selectivity. The present study found that microinjection of ASODN targeted to AT_1 receptor mRNA into the RVLM significantly enhanced the blunted BRS in SHR, suggesting that the attenuated BRS in SHR is related to the enhanced tonic activity of AT_1 receptor in the cell bodies of the RVLM.

Microinjection of both ASDON and losartan into the RVLM had no significant effects on the BRS in normal Wistar rats, but Ang II significantly inhibited the BRS which was abolished completely by pretreatment with losartan. These results indicated that AT_1 receptor activity in the RVLM did not play a tonic role in modulating BRS, and exogenous Ang II in the RVLM inhibited the BRS which is mediated by AT_1 receptor in normotensive rats. An interesting finding in the present study was that bilateral microinjection of Ang II into the RVLM elicited significant inhibitory effects on the BRS in normotensive rats, but failed to depress the BRS

in SHR. These results suggest that the enhanced AT_1 receptor activity evoked by excessive release of Ang II in RVLM was responsible for the blunted BRS in the hypertensive state. The exact mechanism is still not clear. A possible explanation is excessive activity of the intrinsic brain angiotensin system within the RVLM has reduced the BRS to a very low level in SHR, and the AT_1 receptor in the RVLM was in a saturated state because of excessive release of endogenous Ang II. Therefore exogenous Ang II could not depress the BRS any further.

The present study found that blockage of AT_1 receptor or inhibition of AT_1 receptor protein synthesis in the RVLM partially reversed the blunted BRS in SHR, and exogenous Ang II attenuated the BRS only in normotensive rats, indicating that the AT_1 receptor in the RVLM contributes to the blunted BRS in SHR.

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