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Cardiovascular effects of ventrolateral periaqueductal gray (vlPAG) AT1 receptors in normotensive and hemorrhagic rats

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ABSTRACT

Objective(s): The ventrolateral periaqueductal gray (vlPAG) regulates cardiovascular function. Given the presence of Angiotensin II (AngII) and its AT1 receptors (AT1R) in the vlPAG, this study investigated their central and peripheral roles in cardiovascular control during normotensive and hemorrhage (Hem) conditions.

Materials and Methods: Saline, three doses of AngII (0.1, 0.2, and 0.3 nmol) were microinjected into the vlPAG. The AT1R blocker Losartan (Losa) was microinjected alone and before AngII in normotensive and Hem conditions. The peripheral mechanisms of AngII were examined by intravenous injection of hexamethonium (Hexa, a ganglion blocker) and atropine (Atro, a muscarinic receptor blocker), alone and before AngII (0.3 nmol), in both normotensive and Hem conditions. Time course and maximal changes (Δ) of mean arterial pressure (MAP), systolic blood pressure (SBP), and heart rate (HR) were recorded by the PowerLab apparatus and analyzed.

Results: Higher doses of AngII significantly increased HR, SBP, and MAP (P<0.05-P<0.001) than saline. Losa attenuated these effects. Hexa significantly attenuated the pressor effect of AngII (P<0.001), while Atro increased HR (P<0.001). Hem decreased SBP/MAP and increased HR (P<0.01); these responses were augmented by AngII, and Losa blocked this AngII effect. Hexa reduced the cardiovascular improvement induced by AngII during Hem, and Atro enhanced the AngII-induced tachycardia.

Conclusion: AngII in the vlPAG stimulates cardiovascular activity via AT1R in both normotensive and Hem conditions. Furthermore, these peripheral effects of AngII are primarily mediated through sympathetic nervous system activation.

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Introduction

The autonomic nervous system (ANS) plays an essential role in regulating cardiovascular responses during homeostasis and in response to various challenges, such as hemorrhage (Hem). These effects are mediated by numerous brain areas, especially in the brain stem, including the rostral ventrolateral medulla (RVLM), the nucleus tractus solitarius (NTS), the paraventricular nucleus of the hypothalamus (PVN), and the periaqueductal gray matter (PAG) (1, 2). The PAG is one of the most studied of these areas (2). The PAG has several functions, including defense reactions, pain modulation, and cardiovascular regulation (3). Physiological and anatomical studies indicate that the PAG comprises four longitudinal columns: ventrolateral (vlPAG), lateral (lPAG), dorsolateral (dlPAG), and dorsomedial (dmPAG) (3-5). The dlPAG and vlPAG have been most extensively studied in the context of cardiovascular function (3, 6).

Stimulation of the dlPAG has been documented to cause flight-or-fight responses, including sympathoexcitation, pressor effects, and tachycardia. (6). In contrast, the vlPAG is associated with passive coping strategies such as tonic immobility, deep opioid-induced analgesia, and decreased cardiovascular activity (7-10).

The vlPAG receives inputs from the cortex, limbic system, hypothalamus, and spinal cord, and projects to brain areas involved in circulatory regulation, such as the RVLM (11), the caudal ventrolateral medulla (CVLM), and the caudal midline medulla (CMM) (12, 13). The cardiovascular effect of the vlPAG is complex. Early studies reported that its excitation in anesthetized normotensive animals causes a depressor response and bradycardia, likely via inhibition of RVLM sympathetic premotor neurons (14, 15). However, more recent evidence indicates that vlPAG activation can also produce pressor and tachycardic effects. For example, Matsuyama et al. reported that vlPAG neurons projecting to the RVLM mediate pressor responses during social defeat stress (12, 16). Consistently, we have previously shown that L-glutamate (L-Glu) microinjection into the vlPAG induces pressor effects in both normotensive and hemorrhagic hypotensive rats (17). These results suggest the vlPAG can exert bidirectional control over cardiovascular function, potentially depending on the nature of the stimulus and the

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neural pathways engaged (11).

The vlPAG contains various neurotransmitters, including acetylcholine, glutamate, opioids, and Angiotensin II (AngII) (18, 19). Microinjection of these substances produces diverse cardiovascular effects; for example, acetylcholine decreases blood pressure (20). At the same time, L-Glu increases it (17). The renin-angiotensin system (RAS) is an important system that has numerous physiological and pathological effects in the body (21). The important product of this system is AngII, which is produced by the action of renin and further angiotensin-converting enzyme (ACE) (22). AngII has two receptor subtypes: type 1 (AT1) and type 2 (AT2). It is well known that the AT1 receptor plays an essential role in regulating the central cardiovascular system (21). For example, Sherkat et al. (2023) show that the AT1 receptor in the lateral parabrachial nucleus produces an excitatory effect that is mediated by the sympathetic system (23). In the bed nucleus of the stria terminalis (BST), AngII, via increased vasopressin secretion, increases blood pressure (24), and AngII injection into the PVN enhances blood pressure(25). The presence of AngII and AT1Rs in the vlPAG has been documented, and it is reported that they are involved in pain modulation and anxiety (26-28). However, the specific cardiovascular functions of the AngII/AT1R system in the vlPAG, especially during hemodynamic challenges like Hem, remain unknown.

Hem is a life-threatening condition triggering complex autonomic compensations. The vlPAG is activated during Hem, and its inactivation attenuates hemorrhage-induced hypotension and bradycardia (29, 30). At the same time, our recent work showed that L-Glu injection into the vlPAG induces a pressor effect in both normotensive and hemorrhagic rats (17). This evidence shows that the vlPAG is an important area in the central response to blood loss (16). Although AngII/AT1R is present in the vlPAG, its role in cardiovascular function remains unknown. Therefore, the primary objective of this study was to investigate the central and peripheral cardiovascular effects of AngII and its AT1 receptors in the vlPAG under both normotensive and Hem conditions. To test this, we examined cardiovascular responses to vlPAG microinjections of AngII and Losartan (Losa), the AT1R antagonist, into the vlPAG. Furthermore, we evaluate the peripheral autonomic mechanisms by pre-treating animals with the ganglionic blocker hexamethonium (Hexa) and the muscarinic antagonist atropine (Atro).

Materials and Methods

Animals

This experiment was carried out on eighty-four male Wistar rats (230–290 g). The rats were obtained from the animal house of the Faculty of Medicine, Mashhad University of Medical Sciences (MUMS). The rats were housed under standard conditions (temperature, 21–23 °C; 12-hr light-dark cycle; *ad libitum* feeding and water). The experimental process was carried out in accordance with the guidelines of the MUMS ethics committee (Approval ID: IR.MUMS.AEC.1401.093).

Drugs

The drugs used in this study are as follows: Urethane (anesthetic), AngII (non-selective angiotensin receptor agonist), Losartan (Losar, AT1 receptor antagonist), Atro (antagonist of muscarinic receptors), and Hexa (ganglionic receptor blocker). All drugs were obtained from Sigma.

Experimental groups

The rats were randomly assigned to fourteen groups (n=6 per group), divided into normotensive (Part A) and hemorrhagic (Part B) conditions, as detailed in Table 1.

Arterial cannulation and recording of cardiovascular responses

Initially, anesthesia was induced with an intraperitoneal injection of urethane (1.4 g/kg) (34). Following the confirmation of deep anesthesia, an incision was made in the inner femoral region. The femoral artery was isolated and cannulated with an angiocatheter (22-gauge) filled with heparinized (50 U/ml) saline (35). The catheter was secured and connected to a Power Lab device (AD Instruments, Bella Vista, NSW, Australia) via a pressure transducer for continuous recording of heart rate (HR), mean arterial pressure (MAP), and systolic blood pressure (SBP) (17). A second angiocatheter was inserted into another artery for blood withdrawal. Throughout the experiments, the rat's body temperature was maintained at 37.5°C using a warmer lamp.

Microinjection of drugs

Following arterial cannulation for drug microinjection, rats were placed in a stereotaxic device. After fixing the head, a scalp incision was made to expose the skull and identify the bregma and lambda. The coordination of the vlPAG (AP: -6.6 to -8.7 mm, L: ±0 to 1.5 mm, H: 5.5–6.5 mm from bregma) was determined using the Paxinos and Watson rat brain atlas (36). A 2 mm diameter craniotomy was drilled above the vlPAG, and drugs were microinjected into the vlPAG via a micropipette inserted through the opening. The micropipette was connected to a manual pump (Stoelting, USA) via polyethylene tubing, and the injection was performed manually. Intravenous injections of Atro and Hexa were performed via the tail vein.

Experimental design

A. Experiments in normotensive conditions

In normotensive groups, the cardiovascular parameters were first recorded for a 10 min for stabilization. Following confirmation of stability, a 5-minute baseline recording was taken. Subsequently, drugs were microinjected into the vlPAG, and HR, MAP, and SBP were recorded for up to 30 min. Initially, doses of 0.1, 0.2, and 0.3 nmol of AngII were administered to different groups to identify the most effective dose for use in subsequent experiments. Next, Losa was microinjected into the vlPAG, alone and again 5 min before AngII microinjection, with cardiovascular responses recorded for 30 min. To evaluate the role of the ANS in the cardiovascular effect of AngII of the vlPAG, the ganglionic blocker Hexa was injected intravenously, both alone and 5 minutes before the vlPAG AngII injection. Finally, to assess the specific contribution of the parasympathetic system, Atro (a non-selective muscarinic receptor antagonist; 1 mg/kg, IV) was injected (37). Five minutes later, AngII was microinjected into the vlPAG, and cardiovascular parameters were recorded.

B. Experiments in Hemorrhagic Conditions

In Hem groups, after hemodynamic stabilization, approximately 15% of the total blood volume (TBV) was

Table 1. Experimental groups following microinjection of AngII into the vlPAG in normotensive and hemorrhagic hypotensive rats

Condition	Group name	vlPAG Microinjection (after stabilization/Hem)	Intravenous injection
A (Normotensive)	1. Saline	Saline	-
	2. AngII 0.1	AngII (0.1 nmol)	-
	3. AngII 0.2	AngII (0.2 nmol)	-
	4. AngII 0.3	AngII (0.3 nmol)	-
	5. Losa	Losartan (20 nmol)	-
	6. Losa+AngII	Losartan (20 nmol) + AngII (0.3 nmol, 5 min later)	-
	7. Atro+AngII	AngII (0.3 nmol)	Atro (1 mg/kg)
	8. Hexa+AngII	AngII (0.3 nmol)	Hexa (10 mg/kg)
B (Hemorrhagic)	9. Hem+Saline	Saline	-
	10. Hem+AngII	AngII (0.3 nmol)	-
	11. Hem+Losa	Losartan (20 nmol)	-
	12. Hem+Losa+AngII	Losartan (20 nmol) + AngII (0.3 nmol, 5 min later)	-
	13. Hem+Atro+AngII	AngII (0.3 nmol)	Atro (1 mg/kg)
	14. Hem+Hexa+AngII	AngII (0.3 nmol)	Hexam (10 mg/kg)

Doses of drugs were selected based on previous studies (23, 31, and 32). The volume of microinjection into the vlPAG for all drugs was 100 to 150 nanolite and intravenous injection volume was 0.4 ml (23, 33). vlPAG: ventrolateral periaqueductal gray, Hexa: Hexamethonium, Atro: Atropine , AngII: Angiotensin II , Hem: Hemorrhage

withdrawn at a rate of 1 ml/100 g body weight over 10 min. TBV was calculated using the formula: TBV: 0.06 ml/g body weight + 0.77(38). This method induced a moderate Hem, reducing SBP by approximately 30–35 mmHg, indicating the early compensatory phase (17, 38). Following Hem induction, saline, AngII, and Losa (both alone and in combination) were microinjected into the vlPAG. To examine the peripheral effect of AngII, Hexa and Atro were injected intravenously, and after 5 min, AngII was microinjected into the vlPAG, and changes in cardiovascular parameters were evaluated (17).

Injection site verification

At the end of the experiments, rats were sacrificed via carbon dioxide inhalation, and their brains were removed and fixed in 10% formalin for 24 hr. Serial 60-µm coronal sections were cut using a microtome (ESM Co., USA) and stained with Cresyl Violet. The location of the microinjections was confirmed using a light microscope according to the atlas of Paxinos and Watson. Data from rats with injection sites located outside the vlPAG were excluded from the analysis (approximately 10% of the total animals).

Statistical analysis

Cardiovascular parameters, including HR, MAP, and SBP, were recorded continuously, and those changes (Δ; the difference between before and after injection) were calculated and presented as mean ± standard error of the mean (SEM).

The statistical analysis of the data was performed using SPSS version 26. The normality of the data was assessed using the Kolmogorov-Smirnov test. Analysis of time course changes (parameters in multiple time points) was performed by repeated-measures ANOVA and Tukey's *post hoc* test. The maximal changes (Δ; Maximal difference between pre- and post-injection of the drug) were analyzed by one-way ANOVA and Tukey's *post hoc* test. A *P*-value < 0.05 was considered statistically significant.

Results

The circulatory effect of normal saline microinjected into the vlPAG region in normotensive rats

After cardiovascular parameters stabilized, saline was microinjected into the vlPAG. The time course and maximal changes in cardiovascular responses were assessed. Baseline values (mean ± SEM) before injection were as follows: (MAP: 110.06 ± 3.1 mmHg, SBP: 127.13 ±4.3 mmHg, and HR: 386.34 ±17.26 beats/min. After microinjection of saline, MAP, SBP, and HR values reached 108.88 ±2.9 mmHg, 124.92 ±4.2 mmHg, and 385.25 ±17.05 beats/min, respectively, which were not significantly different from pre-injection.

Effect of three doses of angiotensin II microinjection into the vlPAG region in normotensive rats

In this part of the study, the circulatory effects of three different doses (0.1, 0.2, and 0.3 nmol) of AngII

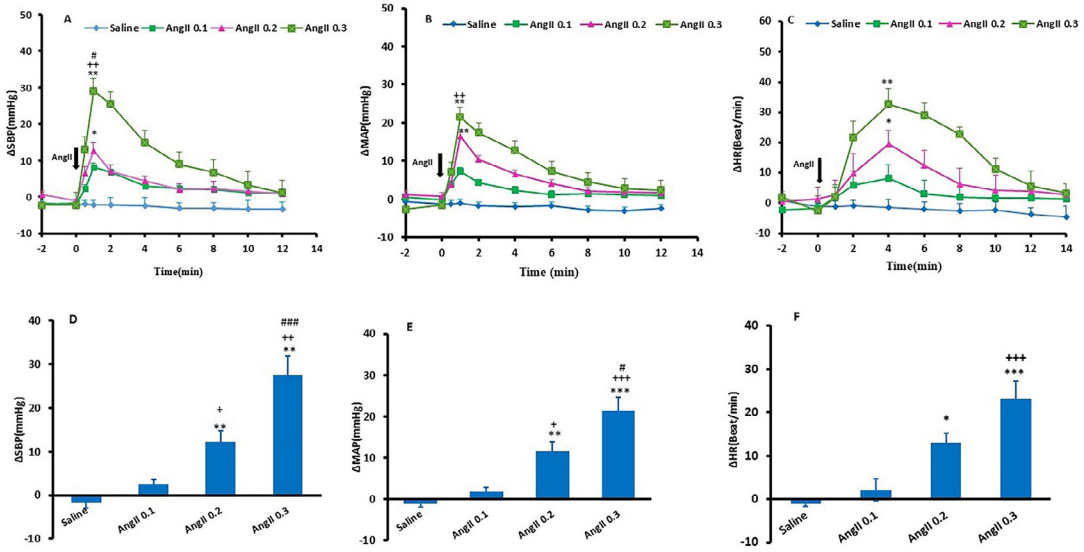

Figure 1. Cardiovascular effects of microinjection of AngII into the vlPAG in anesthetized rats
Time-course (A–C) and maximal changes (D–F) of SBP, MAP and HR following microinjection three doses of AngII (0.1, 0.2 and 0.3 nmol) compared with saline.
Data were expressed as mean ± SEM; n= 6 (repeated measures ANOVA and one-way ANOVA with Tukey's *post hoc* test). Δ: Difference between pre and post injection, AngII: Angiotensin II, MAP: Mean arterial pressure, SBP: Systolic blood pressure, HR: Heart rate. *: P<0.05, **: P<0.01 and ***: P<0.001 vs saline group, +: P<0.05, ++: P<0.01 and +++: P<0.001 vs AngII 0.1 group, #: P<0.05 and ###: P<0.001 vs AngII 0.2 group

microinjected into the vlPAG were examined. Figure 1 shows time-course changes of ΔMAP, ΔSBP, and ΔHR. As has been indicated, doses 0.2 and 0.3 of AngII significantly increase ΔMAP and ΔSBP compared to the control group over time (P<0.05, P<0.1, respectively), while dose 0.1 of AngII did not induce a significant increase. ΔHR increased in response to all three doses of AngII, with doses of 0.2 (P<0.05) and 0.3 (P<0.01) being significant compared to the control group over time (Figure 1C). In comparison of three doses of AngII, all parameters (ΔMAP, ΔSBP, and ΔHR) in the dose of 0.3 nmol were significantly greater with respect to the dose of 0.1 in overtime (P<0.01). Also, ΔSBP in dose 0.2 nmol was significantly lower in comparison with dose 0.3nmol. (P<0.05, Figure 1A, B).

The maximal effects of the three AngII doses were also determined and statistically analyzed. As shown in Figure 1(D-F), ΔMAP, ΔSBP, and ΔHR were not significantly increased by the 0.1 nmol dose of AngII. In contrast, changes induced by 0.2 and 0.3 doses were significantly different from those induced by saline (P<0.01-P<0.001). The highest effects were observed with the 0.3 nmol dose of AngII, which significantly increased SBP, MAP, and HR compared to both the control group and other AngII doses (Figure 1D, E, F).

Effect of losartan alone and Losa+ AngII microinjection into the vlPAG region on cardiovascular responses in normotensive rats

To investigate the role of AT1 receptors in the vlPAG region, Losa was microinjected alone into this area, and its effects on cardiovascular parameters were assessed over time and as maximal changes. The results demonstrated that Losa alone had no significant impact on ΔSBP, ΔMAP, and ΔHR compared to the control group over

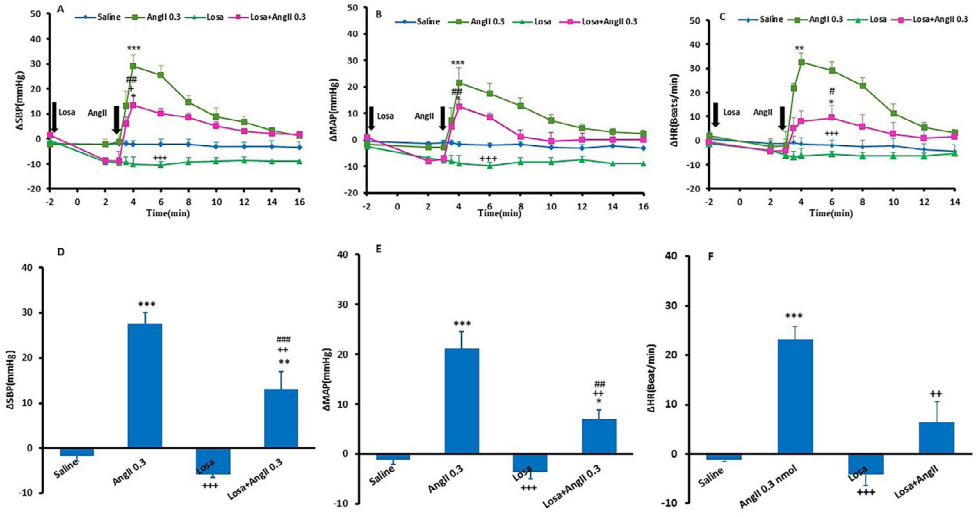

Figure 2. Cardiovascular effects of microinjection of Losa alone and Losa+AngII into the vlPAG in anesthetized rats
Time-course (A–C) and maximum changes (D–F) of SBP, MAP and HR following microinjection Losa and Losa+ AngII compared with AngII alone.
Data were expressed as mean ± SEM; n= 6; Repeated measures ANOVA and one-way ANOVA with Tukey's *post hoc* test were used for time course and maximal changes, respectively. Δ: Difference between pre and post injection, vlPAG: Ventrolateral periaqueductal gray, AngII: Angiotensin II, MAP: Mean arterial pressure, SBP: Systolic blood pressure, HR: Heart rate. Losa: Losartan. *: P<0.05, **: P<0.01 and ***: P<0.001 vs saline group, +: P<0.05, ++: P<0.01 and +++: P<0.001 vs AngII group, #: P<0.05 and ###: P<0.001 vs Losa group

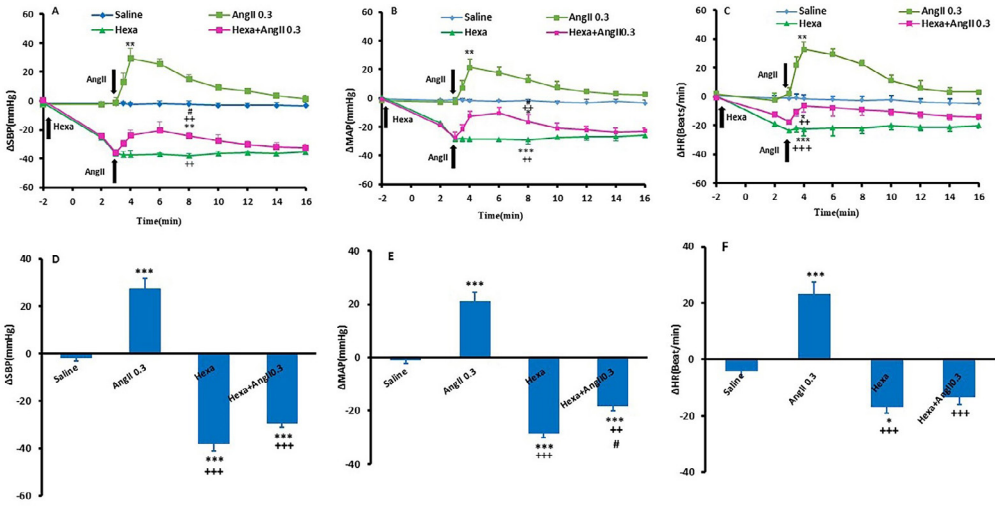

Figure 3. Effects of Hexa pretreatment on cardiovascular responses to microinjections of AngII into the vlPAG in normotensive rats
Time-course (A–C) and maximum changes (D–F) of SBP, MAP and HR following intravenous administration of Hexa alone or prior to AngII. Data were expressed as mean ± SEM; n= 6; Repeated measures ANOVA and one-way ANOVA with Tukey's *post hoc* test were used for time course and maximal changes, respectively. Δ: Difference between pre and post injection, vlPAG: Ventrolateral periaqueductal gray, AngII: Angiotensin II, MAP: Mean arterial pressure, SBP: Systolic blood pressure, HR: Heart rate. Hexa: Hexamethonium ***P<0.001, **P<0.01, *P<0.05 vs Saline; +++P<0.001, ++P<0.01 vs AngII; #P<0.05 vs Hexa

time (Figure 2A, B, and C). To further evaluate the AT1 receptor function, in a separate group, first received a Losa microinjection, followed 5 min later by AngII (0.3 nmol) microinjected into the vlPAG (Losa + AngII group). The pressor and tachycardia responses (ΔSBP, ΔMAP, ΔHR) in this group were significantly attenuated compared to the AngII-alone group over time (P<0.05 to P<0.01, repeated-measures ANOVA; Figure 2). However, the cardiovascular effects of the AngII group were not completely blocked by Losa pretreatment. Both ΔSBP and ΔMAP in the Losa +AngII group remained significantly elevated compared to the saline group over time (P<0.01, repeated measures ANOVA). Also, the increases in SBP and MAP (P<0.01) in the Losa +AngII group were significantly greater than in the Losa-alone group (Figure 2 A, B). The maximal effect also confirmed these findings. Losa alone produced no significant effect on any parameter compared to the saline group. However, when microinjected before AngII, it significantly attenuated the cardiovascular effect of AngII. As shown in Figure 2. ΔSBP and ΔMAP in the Losa + AngII group were significantly lower than in both the AngII and the saline alone groups (P<01-P<0.001, one-way ANOVA). The ΔHR in the Losa + AngII group was also significantly lower than in the AngII alone group but did not differ significantly from the saline group (Figure 2D).

The circulatory effect of AngII microinjection in the vlPAG region following hexamethonium intravenous injection in normotensive rats

To examine the peripheral circulatory processes caused by AngII microinjection into the vlPAG, ganglia of the ANS were blocked by intravenous injection of Hexa, and changes in responses were examined at several times. Time course changes show that Hexa injection significantly decreased ΔSBP, ΔMAP, and ΔHR with respect to saline in several times (P<0.001; repeated measures ANOVA, Figure 3A, B, C). In another group, the first Hexa (IV) was injected, and after 5 min, AngII was microinjected into the vlPAG (Hexa + AngII group). In this group, changes in ΔSBP, ΔMAP, and ΔHR induced by AngII are significantly attenuated by Hexa at several time points (P<0.001, repeated-measures ANOVA; Figure 3). Nevertheless, ΔMAP (P<0.05) and ΔHR (P<0.05) were significantly increased compared to Hexa alone over time (Figure 3 B, C). In the Hexa + AngII group, also ΔSBP, ΔMAP, and ΔHR were significantly less than those of the control groups over time (P<0.05-P<001, repeated measures ANOVA, Figure 3A, B, C). The maximal responses were also calculated and analyzed. Figure 3(D-F) shows that after injection of Hexa, maximal cardiovascular parameters (ΔSBP (P<0.001), ΔMAP (P<0.001), and ΔHR (P<0.05) significantly decreased compared to saline and AngII. In the Hexa+AngII group, Hexa could also attenuate the effect of AngII, so all parameters were significantly lower than AngII alone (P<0.01 –P<0.001, Figure 3D, E, F).

Circulatory effects of AngII microinjection into the vlPAG following atropine intravenous injection in normotensive rats In this part of the study, the potential influence of the parasympathetic system on the cardiovascular effects of AngII microinjected into the vlPAG was investigated. For this purpose, Atro (1 mg/kg; IV) was microinjected first. Repeated-measures ANOVA analysis showed that intravenous Atro injection alone did not insignificantly alter ΔSBP and ΔMAP over time (P>0.05, Figure 4A, B) but did produce an increasing ΔHR over time (P<0.001, Figure 4C).

In a separate group, Atro was administered intravenously, followed 5 min later by microinjection of AngII into the vlPAG (Atro + AngII group). In the presence of Atro, the pressor effect of AngII was insignificantly reduced compared to the AngII alone group over time (Figure 4A and B), while the ΔHR response was significantly potentiated (P<0.05, Figure 4C). A comparison of the Atro alone group with the Atro + AngII group shows that ΔSBP and ΔMAP in the Atro + AngII group were significantly greater than those in the Atro group alone over time (P<0.01, Repeated-measures ANOVA; Figure 4A and B). The ΔHR in the Atro+AngII group was also significantly higher with respect to the Atro alone group (P<0.05, Figure 4C). Furthermore, all cardiovascular parameters in the Atro +AngII group were significantly greater compared to the control group

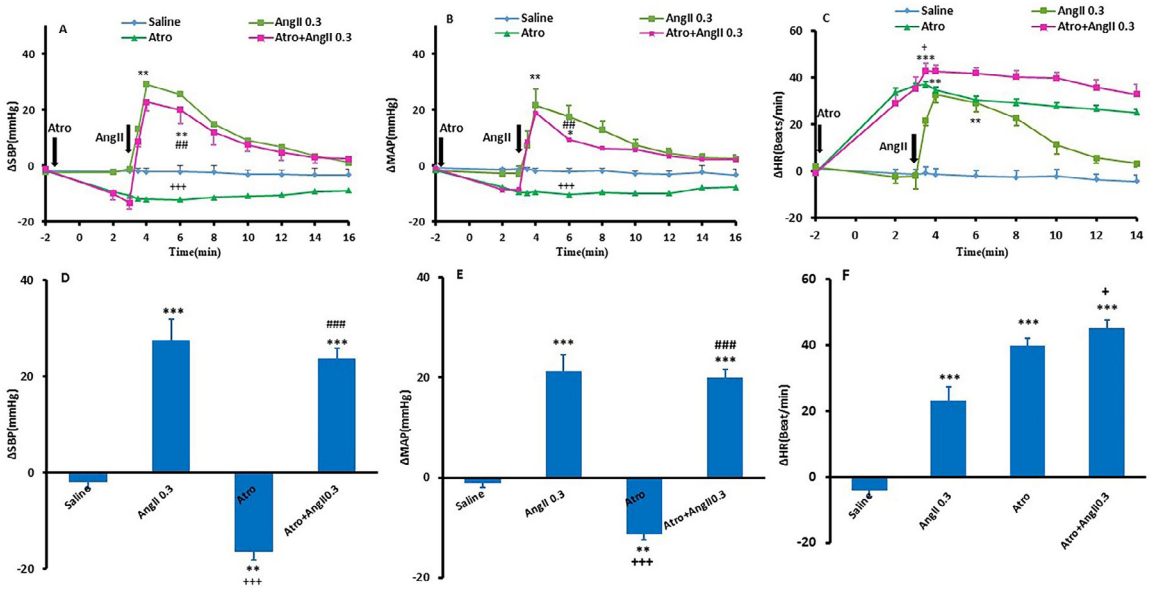

Figure 4. Effects of Atro pretreatment on cardiovascular response to microinjections of AngII into the vlPAG in normotensive rats
Time-course (A–C) and maximum changes (D–F) of SBP, MAP and HR following intravenous administration of Atro alone or prior to AngII. Data were expressed as mean ± SEM; n= 6; Repeated measures ANOVA and one-way ANOVA with Tukey's *post hoc* test were used for time course and maximal changes, respectively. Δ: Difference between pre and post injection, vlPAG: Ventrolateral periaqueductal gray, AngII: Angiotensin II, MAP: Mean arterial pressure, SBP: Systolic blood pressure, HR: Heart rate, Atro: Atropine
***P<0.001, **P<0.01, *P<0.05 vs Saline; +++P<0.001, ++P<0.01 vs AngII; ###P<0.001, ##P<0.01 vs Atro

(P<0.001; Figure 4).

The maximal responses also confirmed these findings. Atro (IV) alone produced a non-significant reduction in ΔSBP and ΔMAP but caused significant tachycardia (P<0.001). While Atro did not significantly attenuate the pressor effect of AngII, it potentiated the AngII-induced increase in ΔHR (P<0.05, Figure 4D, E, F).

Circulatory effects of saline microinjection into the vlPAG in hemorrhagic rats

In this study, Hem was induced by withdrawing approximately 15% of the animal's blood volume, a quantity calculated in advance. This volume of blood loss could reduce the MAP by 35-40 mmHg. As shown in Figure 5, Hem resulted in a significant decrease in ΔSBP and ΔMAP over time compared with the non-hemorrhaged saline control group (P<0.01, repeated-measures ANOVA; Figure 5A and 5 B). Concurrently, ΔHR was significantly increased (P<0.01, Figure 5C). Microinjection of saline into the vlPAG following Hem (Hem +saline group) had no significant effect on ΔSBP, ΔMAP, and ΔHR over time compared to the Hem alone group.

Maximal changes confirmed these findings. In the Hem group, both ΔMAP and ΔSBP were significantly lower (P<0.001, Figure 5D and E, one-way ANOVA) and HR was

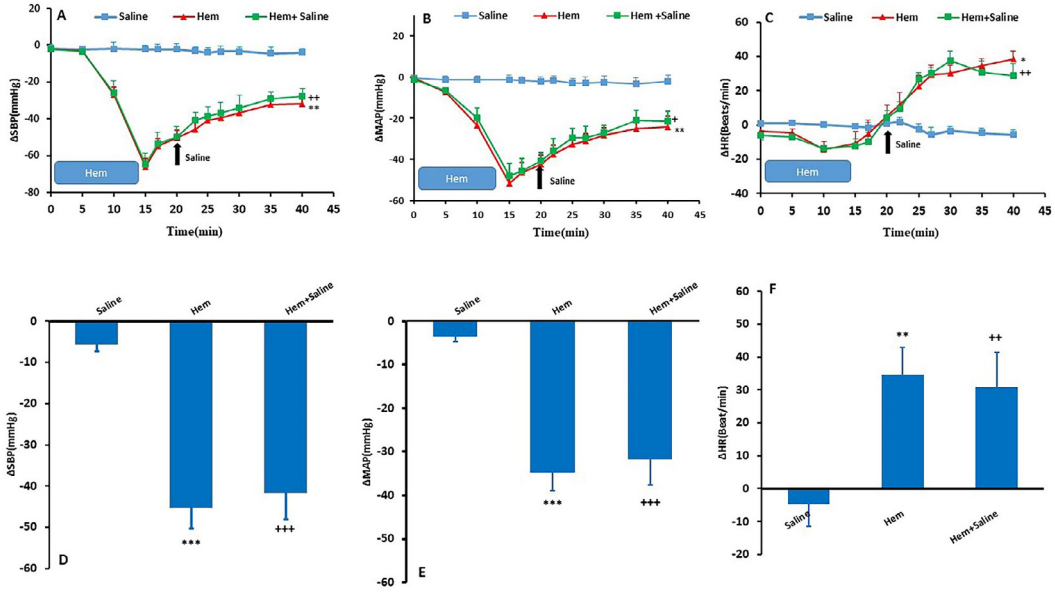

Figure 5. Cardiovascular responses in hemorrhagic hypotensive rats
Time-course (A–C) and maximum changes (D–F) of SBP, MAP and HR following saline microinjection into the vlPAG in hypotensive rats. Data were expressed as mean ± SEM; n= 6; Repeated measures ANOVA and one-way ANOVA with Tukey's *post hoc* test were used for time course and maximal changes, respectively.
Δ: Difference between pre and post injection, vlPAG: Ventrolateral periaqueductal gray, AngII: Angiotensin II, MAP: Mean arterial pressure, SBP: Systolic blood pressure, HR: Heart rate, Hem: Hemorrhage.***P<0.001, **P<0.01 Hem vs Saline, ++P<0.01, +P<0.05 Hem + Saline vs Saline

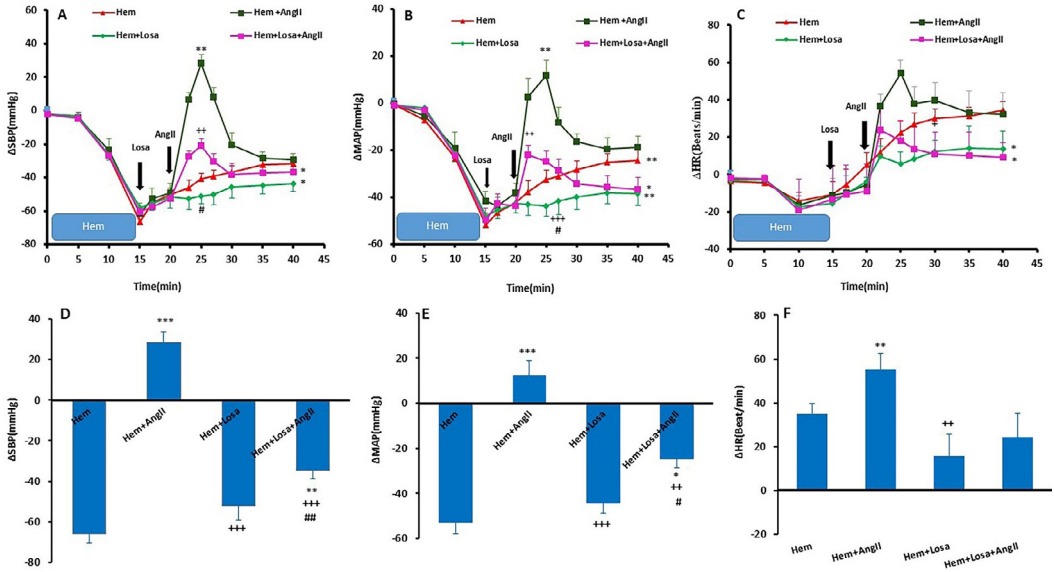

Figure 6. Cardiovascular effects of microinjection of Losa alone and Losa+AngII into the vlPAG in hypotensive rats
Time-course (A–C) and maximum changes (D–F) of SBP, MAP and HR following microinjection AngII, Losa and Losa+ AngII in hemorrhage condition. Data were expressed as mean ± SEM; n= 6; Repeated measures ANOVA and one-way ANOVA with Tukey's *post hoc* test were used for time course and maximal changes, respectively. Δ: Difference between pre and post injection, vlPAG: Ventrolateral periaqueductal gray, AngII: Angiotensin II, MAP: Mean arterial pressure, SBP: Systolic blood pressure, HR: Heart rate, Losa: Losartan, Hem: Hemorrhage. ***P<0.001, **P<0.01, *P<0.05 vs Hem, +++P<0.001, ++P<0.01, +P<0.05 vs Hem +AngII, ##P<0.01, #P<0.05 vs Hem+ Losa

significantly higher (P<0.01, one-way ANOVA; Figure 5F) than in the saline group.

Effect of microinjection of AngII, losartan, and losartan + AngII into the vlPAG on cardiovascular responses in hemorrhagic Rats

Following the induction of Hem, AngII (0.3 nmol) was microinjected into the vlPAG area. AngII attenuated hemorrhage-induced hypotension, as evidenced by a significant increase in the time course of ΔSBP and ΔMAP compared with the Hem group (P<0.01; Figure 6A and B; repeated-measures ANOVA). The ΔHR also increased, but this effect did not differ significantly from that of the Hem group over time (Figure 6C). To evaluate the role of the AT1 receptor in the Hem condition, Losa was microinjected into the vlPAG first, followed by AngII (0.3 nmol) 5 min later (Losa + AngII group). Losa blocked the improvement in cardiovascular responses during Hem.

As shown in Figure 6, the time course changes of ΔSBP and ΔMAP in the Losa +Hem group were significantly lower than in the Hem alone group over time (P<0.05-P<0.01, Figure 6 A, B). Also, the tachycardia induced by Hem was attenuated by Losa over time (P<0.05; Figure 6C). In the Hem +Losa + AngII group, Losa pretreatment attenuated the cardiovascular effects of AngII microinjected into the vlPAG. Time-course analysis revealed that ΔSBP and ΔMAP in this group were significantly lower in the Hem +AngII group over time (P<0.01, Figure 6 A, B). In contrast, the changes in ΔHR were not significantly different between these two groups (Figure 6C). The maximal responses further demonstrated that ΔSBP (P<0.001), ΔMAP (P<0.001), and ΔHR (P<0.05) in the Hem +AngII group were significantly elevated compared to the Hem alone group (Figure 6D, E, and F). Microinjection of Losa (Losa+Hem group) significantly decreased ΔSBP, ΔMAP, and ΔHR compared to the Hem alone group (P<0.05, one-way ANOVA, Figure 6 D, E, F). In the Hem +Losa + AngII group, Losa significantly attenuated the pressor effect of AngII, resulting in ΔSBP and ΔMAP that were significantly lower than in the AngII alone group (P<0.001, Figure 6 D, E, one-way ANOVA). Similarly, ΔHR in this group was significantly lower than in the AngII alone group (P<0.01, Figure 6F).

Effect of AngII microinjection into the vlPAG following intravenous injection of hexamethonium in hemorrhagic rats

To investigate the peripheral effects of AngII in the Hem condition, rats were subjected to Hem, followed by intravenous injection of the ganglion blocker (Hexa) and subsequent microinjection of AngII into the vlPAG.

The Time course analysis showed that Hexa injection significantly decreased ΔSBP, ΔMAP, and ΔHR over time compared to the Hem alone group (P<0.05- P<0.01, Figure 7 A, B, C). In the Hem+Hexa+AngII group, ΔSBP, ΔMAP, and ΔHR were significantly lower than in the Hem+AngII group over time (P<0.01, Figure 7 A, B, C). Furthermore, ΔSBP and ΔMAP in the Hem+Hexa+AngII group were significantly higher than in the Hem alone group (P<0.05, Figure 7A, B), while ΔHR was significantly lower (P<0.05, Figure 7C). All parameters in the Hem +Hexa +AngII group were also significantly higher than in the Hexa alone group (P<0.01).

Analysis of maximal responses indicated that ΔSBP and ΔMAP in the Hem+ Hexa group were not significantly different from the Hem group (P>0.05, Figure 7 D, E), while ΔHR was significantly altered in the group (P<0.001, Figure 7 F). AngII microinjection reversed the hypotension induced by Hem and Hexa. Consequently, in the Hem+Hexa+AngII group, ΔSBP and ΔMAP were significantly Higher than in both the Hem and Hem +Hexa groups (P<0.01, one-way ANOVA, Figure 7 D, E). The ΔHR in the Hem+ Hexa +AngII group was significantly attenuated compared to Hem +AngII (P<0.01) and not significantly different from the Hem +Hexa (Figure 7F).

Effect of AngII microinjection into the vlPAG following intravenous injection of atropine in hemorrhagic rats

To investigate the role of the parasympathetic system in the cardiovascular effects of AngII in the vlPAG during Hem, animals were first subjected to Hem, then to intravenous

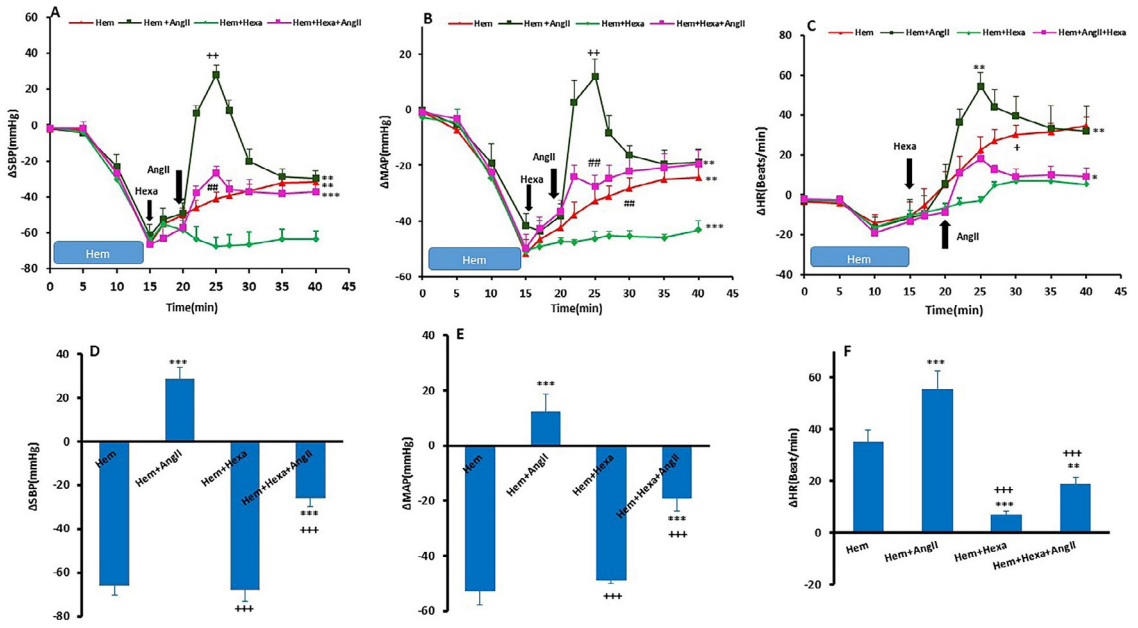

Figure 7. Effects of Hexa pretreatment on cardiovascular responses to microinjections of AngII into the vlPAG in hypotensive rats
Time-course (A–C) and maximum changes (D–F) of SBP, MAP and HR following intravenous administration of Hexa alone or prior to AngII in hemorrhage condition. Data were expressed as mean ± SEM; n= 6; Repeated measures ANOVA and one-way ANOVA with Tukey's *post hoc* test were used for time course and maximal changes, respectively. Δ: Difference between pre and post injection, vlPAG: Ventrolateral periaqueductal gray, AngII: Angiotensin II, MAP: Mean arterial pressure, SBP: Systolic blood pressure, HR: Heart rate. Hexa: Hexamethonium. ***P<0.001, **P<0.01, *P<0.05 vs Hem group, +++P<0.001, ++P<0.01, +P<0.05 vs Hexa group, ###P<0.001, ##P<0.01 vs Hem + AngII group

Atro, and subsequently to AngII microinjection.

Time course changes (Figure 8A, B, and C) showed that intravenous Atro injection alone caused a non-significant decrease in ΔSBP and ΔMAP over time (P>0.05, repeated-measures ANOVA) but a significant increase in ΔHR (P<0.01, repeated-measures ANOVA). In the presence of Atro, administration of AngII (Hem+Atro+AngII group) resulted in a decrease in ΔSBP and ΔMAP and an increase in ΔHR compared to the AngII alone (Hem+AngII group). However, these changes over time were not statistically significant (Figure 8 A, B, C).

Analysis of the maximal changes (Figure 8D, E, F) showed that during Hem, Atro injection did not significantly alter ΔSBP, ΔMAP, or ΔHR from the Hem group alone. In contrast, AngII microinjection following Atro (Hem+Atro+AngII group) significantly increased both ΔSBP (P<0.001) and ΔMAP (P<0.01) compared to the Hem alone and Atro alone groups (Figure 8D, E). The ΔHR in

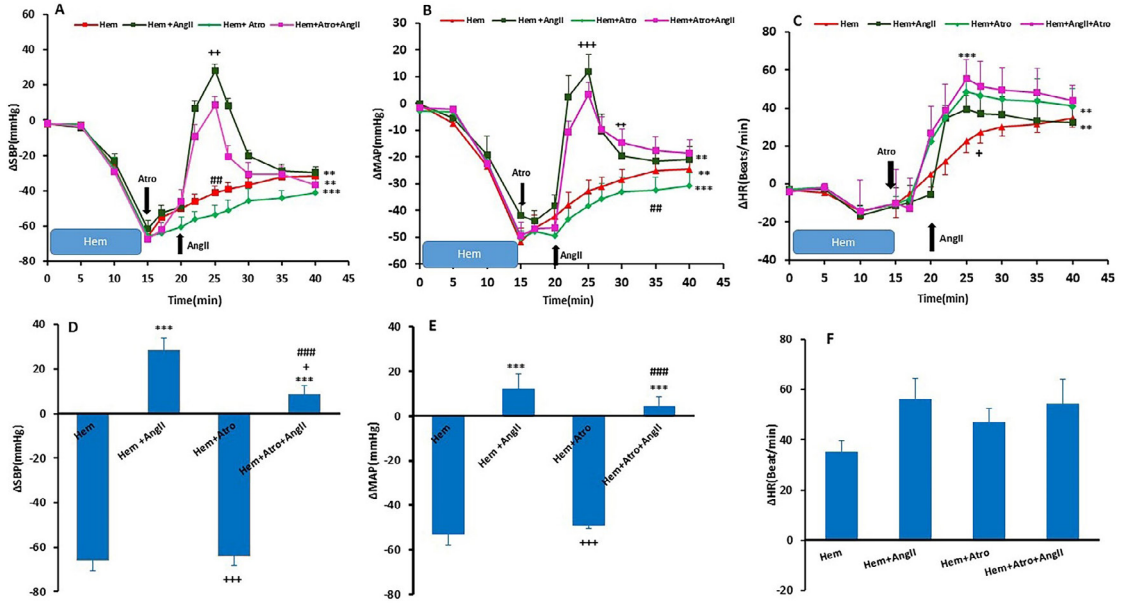

Figure 8. Effects of Atro pretreatment on cardiovascular responses to microinjections of AngII into the vlPAG in hypotensive rats
Time-course (A–C) and maximum changes (D–F) of SBP, MAP and HR following intravenous administration of Atro alone or prior to AngII in hemorrhage condition. Data were expressed as mean ± SEM; n= 6; Repeated measures ANOVA and one-way ANOVA with Tukey's *post hoc* test were used for time course and maximal changes, respectively. Δ: Difference between pre and post injection, vlPAG: Ventrolateral periaqueductal gray, AngII: Angiotensin II, MAP: Mean arterial pressure, SBP: Systolic blood pressure, HR: Heart rate, Atro: Atropine. ***P<0.001, **P<0.01, *P<0.05 vs Hem group, +++P<0.001, ++P<0.01 vs Hem+Atro group, ###P<0.001, ##P<0.01 Hem vs Hem + Atro group

the Hem +Atro+AngII group was not significantly different from that in the AngII or Atro alone groups. (Figure 8 F).

Discussion

The present study provides novel evidence that AngII microinjected into the vlPAG elicits dose-dependent pressor and tachycardia responses in both normotensive and Hem conditions. The key findings are: 1) this effect is mediated primarily by AT1Rs, as it was attenuated by Losa; 2) the peripheral pathway requires autonomic ganglionic transmission, and 3) the sympathetic nervous system is the dominant autonomic effector.

Ang II, the primary effector peptide of the RAS, is an essential central regulator of cardiovascular function, acting mainly through the AT1 receptor (17, 22, 23, 32). While the peripheral actions of AngII are well established (39), its central effects on cardiovascular regulation have attracted increasing attention. Beyond circulating, AngII is also synthesized locally within the brain by intrinsic RAS components, where it participates in autonomic and cardiovascular control (40). Consistent with this, microinjection of AngII into the cardiovascular-related centers such as the paraventricular nucleus (PVN), the organum vasculosum of the lamina terminalis (OVLT), and the bed nucleus of the stria terminalis (BST), has been shown to evoke pressor responses and increase sympathetic nerve activity (41, 42). The important receptor involved in the cardiovascular effects of AngII is the AT1R (42), which likely acts through mechanisms such as increased sympathetic outflow, modulation of baroreflex sensitivity, and promotion of vasopressin secretion and fluid balance (32, 43). Consistent with these previous findings, our results demonstrate that AngII microinjection into the vlPAG also elicits significant cardiovascular excitation. The fact that this response was markedly attenuated by Losa confirms that it is predominantly mediated by AT1Rs within the vlPAG, thereby identifying this midbrain structure as a novel site for the excitatory cardiovascular actions of central AngII. The mechanism by which AngII exerts this effect in the vlPAG remains unknown. The vlPAG is a critical region for integrating autonomic and behavioral responses to stress and threat, which are often categorized as active or passive coping strategies. Active strategies, mediated by the lateral, dorsolateral, and dorsomedial PAG, typically increase cardiovascular activity. In contrast, passive coping strategies, orchestrated by the vlPAG, involve freezing, tonic immobility, hypotension, and bradycardia (44, 45). Consistent with this, Henderson *et al.* (1997) reported that vlPAG excitation induces hypotension and bradycardia (46). Despite the role of the vlPAG in the hypotensive effect, we observed that microinjection of AngII into the vlPAG induced a pressor response, suggesting a more complex role for this nucleus. This finding is consistent with other studies reporting stimulation-induced pressor responses. For example, microinjection of noradrenaline (47) or L-Glu (17) into the vlPAG has been shown to increase BP. Therefore, vlPAG can have different effects on cardiovascular activity, possibly depending on the neurochemical background and the specific neural pathways involved.

The mechanisms and pathways involved in these opposing cardiovascular responses (hypotension and hypertension) are not fully defined. However, evidence suggests that the cardiovascular effects of the vlPAG are primarily mediated by its projections to brain regions involved in cardiovascular regulation, such as the RVLM, a major sympathetic premotor region (13, 48). These projections from the vlPAG to the RVLM can be both direct and indirect (46).

It is proposed that during less vigorous stimuli, such as mild stress and threat, mild pain, or the compensatory phase of Hem, the direct excitatory projection from vlPAG to RVLM is activated. This leads to sympathetic excitation, resulting in increased blood pressure and HR. In contrast, during vigorous stimulus or inescapable stimuli such as deep and prolonged pain (13, 49) or the decompensatory phase of Hem (approximately 30% total blood volume loss) (30), both direct and indirect projections are activated. Because the effect of indirect is more vigorous, the co-activation of these opposing pathways produces net hypotension and bradycardia.

Two indirect pathways are suggested: a) The vlPAG-CMM-RVLM pathway, which is an essential indirect projection from the vlPAG to the CMM(50). The CMM contains serotonergic neurons that project to the RVLM and IML of the spinal cord (46). These neurons inhibit sympathetic activity and suppress RVLM neuron firing, thereby reducing blood pressure. b) The vlPAG- CVLM-RVLM pathway that vlPAG neurons project to the CVLM (46, 51). The CVLM contains GABAergic neurons that project to and inhibit vasomotor neurons in the RVLM, thereby causing hypotension and bradycardia (3, 51).

The specific neuronal types involved in these direct and indirect projections are not entirely defined. However, our previous study demonstrated that L-Glu injection into the vlPAG produces pressor and tachycardic effects (17). This suggests that the direct vlPAG - RVLM pathway mediating this response is likely glutamatergic. Given that the cardiovascular effect of AngII observed in the present study is similar to that of L-Glu, it is plausible that AngII acts via the same pathway. Furthermore, it has been shown that RAS and AngII are produced locally in various brain regions, including the paraventricular nucleus (PVN) (52), where they interact with L-Glu to potentiate cardiovascular responses (40). Therefore, we propose that in the vlPAG, AngII may act synergistically with glutamate—a mechanism previously reported in the PVN (Young and Davisson, 2015)—to excite neurons that project directly to the RVLM.

Alternatively, a non-exclusive adrenergic signaling within the vlPAG has also been shown. It was reported that microinjection of norepinephrine into the vlPAG, which increased vasopressin secretion, produced a pressor effect (47). Therefore, the cardiovascular effects of AngII may also be mediated by activation of local adrenergic neurons that stimulate vasopressin release. However, future studies are needed to confirm this opinion.

To examine the peripheral cardiovascular mechanisms of AngII in the vlPAG, we first blocked autonomic ganglia by intravenous injection of Hexa. In the presence of Hexa, the cardiovascular effect of AngII was significantly attenuated. This reduction in the AngII-induced pressor response confirms that its central signal is mediated primarily via the ANS. However, since Hexa did not completely abolish the response, we suggest other factors, such as vasopressin secretion, are likely also involved. Because the vlPAG has projections to the hypothalamus(44), we recommend that this pathway may be necessary for stimulating vasopressin secretion.

To distinguish the roles of the sympathetic and parasympathetic nervous systems in the vlPAG-mediated

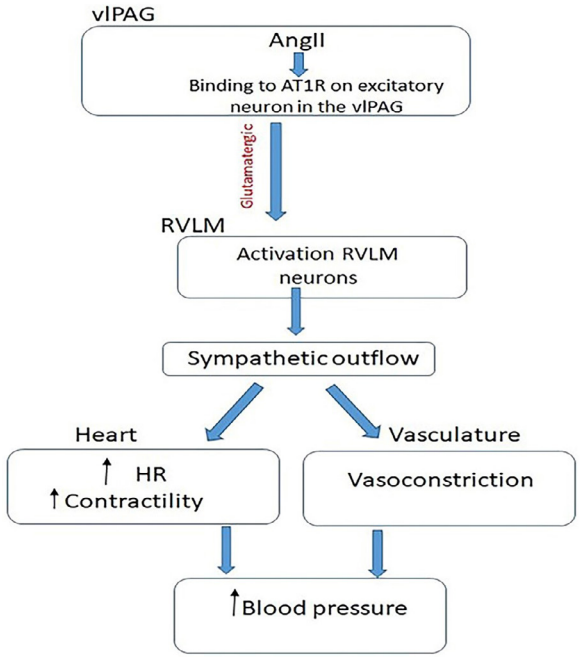

Figure 9. Schematic Illustration of angiotensin II–AT1R signaling in the vlPAG and its cardiovascular effects in rats
RVLM: Rostral ventrolateral Medulla, HR: Heart rate, AngII: Angiotensin II, AT1R: Angiotensin II receptor type 1, vlPAG: Ventrolateral periaqueductal gray

AngII response, the muscarinic receptor blocker (Atro) was administered intravenously, both alone and prior to AngII. While Atro did not significantly attenuate the pressor effect of AngII, it potentiated the tachycardia. This finding indicates a minimal role for the parasympathetic system in the AngII-induced pressor response in the vlPAG. But the potentiation of HR is likely due to the unmasking of vagal withdrawal following muscarinic receptor blockade. Therefore, our results suggest that sympathetic pathways predominantly mediate the cardiovascular effects of AngII in the vlPAG.

Based on our results and existing literature, we propose a model for AngII-AT1R signaling in the vlPAG (Figure 9). In this model, AngII binds to AT1Rs on excitatory (likely glutamatergic) neurons within the vlPAG. This stimulates a direct projection from the vlPAG to the RVLM. The RVLM, in turn, increases sympathetic outflow to the heart and vasculature, resulting in increased HR and blood pressure. This pathway is operational in both normotensive states and during the compensatory phase of hypotension induced by Hem.

Our study has several limitations. First, while the Losa blockade strongly implies AT1R involvement, we cannot completely rule out contributions from other AngII receptor subtypes. Second, the potential role of vasopressin secretion as an alternative pathway for the residual pressor effect after ganglionic blockade was not directly measured. Third, the neuronal systems, such as adrenergic neurons, that evoked the expression of AT1Rs in the vlPAG were not examined.

Conclusion

This study's findings indicate that AngII in the vlPAG elicits dose-dependent cardiovascular activity through its AT1R in both normotensive and hemorrhagic hypotension. Additionally, the most effective component of the ANS mediating peripheral AngII cardiovascular responses is the sympathetic system.

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Data Availability

The data used in this study are available from the corresponding author upon request.

Authors' Contributions

E H, R M, and MN S designed the experiments; E H performed the experiments and collected the data; R M and MNS discussed the results and strategy; MN S Supervised, directed, and managed the study; E H, R M, A R, and MN S approved the final version for publication.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

We acknowledge the use of Grammarly to improve the language and readability.

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