Original Research

Cardiac sympathetic afferent reflex response to intermedin microinjection into paraventricular nucleus is mediated by nitric oxide and γ-amino butyric acid in hypertensive rats

Hong Zhou[1,2], Hai-jian Sun[1], Jin-rui Chang[3], Lei Ding[1], Qing Gao[1,2], Chao-shu Tang[4], Guo-qing Zhu[1] and Ye-bo Zhou[1]

[1]Key Laboratory of Cardiovascular Disease and Molecular Intervention, Department of Physiology, Nanjing Medical University, Nanjing 210029, China; [2]Laboratory Center for Basic Medical Sciences, Nanjing Medical University, Nanjing 210029, China; [3]Department of Physiology, Xi'an Medical University, Shanxi 710021, China; [4]Key Laboratory of Molecular Cardiovascular Science, Ministry of Education, Beijing 100191, China

Corresponding author: Ye-Bo Zhou. Email: zhouyebo666@njmu.edu.cn

Abstract

Intermedin (IMD) is a member of calcitonin/calcitonin gene-related peptide (CGRP) and involves in the regulation of cardiovascular function in both peripheral tissues and central nervous system (CNS). Paraventricular nucleus (PVN) of hypothalamus is an important site in the control of cardiac sympathetic afferent reflex (CSAR) which participates in sympathetic over-excitation of hypertension. The aim of this study is to investigate whether IMD in the PVN is involved in the inhibition of CSAR and its related mechanism in hypertension. Rats were subjected to two-kidney one-clip (2K1C) surgery to induce renovascular hypertension or sham-operation (Sham). Acute experiments were carried out four weeks later under anesthesia. The CSAR was evaluated with the renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP) responses to the epicardial application of capsaicin. The RSNA and MAP were recorded in sinoaortic-denervated, cervical-vagotomized and anesthetized rats. Bilateral PVN microinjection of IMD (25 pmol) caused greater decrease in the CSAR in 2K1C rats than in Sham rats, which was prevented by pretreatment with adrenomedullin (AM) receptor antagonist AM22-52, non-selective nitric oxide (NO) synthase (NOS) inhibitor L-NAME or γ-amino butyric acid $(GABA)_B$ receptor blocker CGP-35348. PVN pretreatment with CGRP receptor antagonist CGRP8-37 or $GABA_A$ receptor blocker gabazine had no significant effect on the CSAR response to IMD. AM22-52, L-NAME and CGP-35348 in the PVN could increase CSAR in Sham and 2K1C rats. These data indicate that IMD in the PVN inhibits CSAR via AM receptor, and both NO and GABA in the PVN involve in the effect of IMD on CSAR in Sham and renovascular hypertensive rats.

Keywords: Intermedin, cardiac sympathetic afferent reflex, paraventricular nucleus, hypertension, nitric oxide, γ-amino butyric acid

***Experimental Biology and Medicine* 2014; 239: 1352–1359. DOI: 10.1177/1535370214533882**

Introduction

Intermedin (IMD), a member of the calcitonin gene-related peptide (CGRP) superfamily, is widely distributed in peripheral organs and central nervous system (CNS).[1–4] The receptor system consisting of calcitonin receptor-like receptor (CRLR) and receptor activity-modifying proteins (RAMPs) is shared by IMD, adrenomedullin (AM) and CGRP.[2] IMD is a potent, peripherally hypotensive, neuropeptide known and shares structural and functional homology with AM.[1] In some cases, the effects of IMD on cardiovascular function are even more potent than AM in both periphery and brain.[5–7]

Sympathetic activity is enhanced in patients with hypertension and various animal hypertensive models.[8–12] Sympathetic overdrive contributes to the pathogenesis and progression of hypertension.[13–15] Intervention of the sympathetic overdrive is viewed as an effective antihypertensive strategy.[16–18] Cardiac sympathetic afferent reflex (CSAR), a positive-feedback, sympathoexcitatory cardiovascular reflex, contributes to the sympathetic activation and hypertension.[19,20] Previous studies in our lab have shown that CSAR is enhanced in renovascular hypertensive rats and spontaneously hypertensive rats (SHRs).[9,21] The paraventricular nucleus (PVN) of the hypothalamus involves in the central pathway of CSAR in normal or

hypertensive rats,[22,23] and it is an important integrative site in the control of cardiovascular activity and sympathetic outflow. In the CNS, immunoreactive IMD has been found in the hypothalamus, especially in the PVN.[3,24] Although the direct effects of peripheral or central IMD on cardiovascular function have been documented,[5–7] the site of action, the effect of IMD on CSAR, and its related mechanism in the PVN have not been explored in the normal and renovascular hypertensive rats.

Central nitric oxide (NO), a nonconventional neurotransmitter, has been proposed to be involved in the regulation of cardiovascular functions by affecting sympathetic activity in hypertension.[25–27] IMD increases NO content and NO synthase (NOS) activity in isolated rat aortas,[28] and it produces vasodilation in an endothelial-dependent manner by production of NO in rat main pulmonary arterial rings.[29] The NOS inhibitor L-NAME reduces IMD-induced renal vasodilator response.[6] NO in the PVN decreases arterial pressure and heart rate (HR), and its down-regulation in the PVN was involved in renal-wrap hypertension.[30] In addition, γ-amino butyric acid (GABA) in the PVN has a tonic inhibitory effect on the sympathetic nervous system,[31,32] and in the PVN it participates in the regulation of CSAR.[33] Furthermore, GABA and NO involve in AM-induced depressor effect in the PVN.[34] It is unknown whether NO and GABA in the PVN are involved in the effect of IMD on CSAR. The present study was designed to determine whether IMD in the PVN inhibits the enhanced CSAR, and whether NO and GABA in the PVN involve in the effect of IMD on CSAR in normal and renovascular hypertensive rats.

Materials and methods

Experiments were carried out in male Sprague–Dawley rats weighing 160–180 g. The procedures were approved by the Experimental Animal Care and Use Committee of Nanjing Medical University and complied with the Guide for the Care and Use of Laboratory Animals (the 8th edition, 2011). The rats were maintained on a cycle of 12 h light and 12 h darkness in a temperature-controlled room and provided free access to laboratory chow and water.

Renovascular hypertensive rat model

Goldblatt two-kidney one-clip (2K1C) method was used to induce renovascular hypertension in rats as previously reported.[20] Simply, the rat was anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal [ip]). The right renal artery of rat was partly occluded to induce renovascular hypertension by placing a U-shaped silver clip (internal diameter 0.20 mm). The sham-operated (Sham) rat received similar surgical process except using silver clip. Systolic blood pressure (SBP) of tail artery ≥160 mm Hg in conscious state is set to meet the criterion of hypertension for experiment.[20]

SBP measurements

Simply, in order to minimize the effect of stress-induced SBP fluctuation on rat, SBP measurement was done daily for at least 10 days before 2K1C or sham operation. A non-invasive computerized tail-cuff system (NIBP, AD Instruments, Australia) was used to measure the SBP of tail artery weekly in conscious rat.[20] The SBP was obtained by averaging 10 measurements.

General procedures of acute experiment

Rat was anesthetized with urethane (800 mg/kg) and a-chloralose (40 mg/kg) intraperitoneally at the end of the fourth week after the 2K1C or sham operation and then ventilated mechanically by using a rodent ventilator (model 683, Harved Apparatus Inc, USA). For continuous recording of arterial blood pressure (ABP), mean arterial pressure (MAP) and heart rate (HR), the right carotid artery of rat was cannulated and connected with a pressure transducer (MLT0380, AD Instruments). Bilateral baroreceptor denervation and vagotomy were carried out and identified on rat as previously reported.[35]

Renal sympathetic nerve activity (RSNA) recordings

To isolate the left renal sympathetic nerve, a retroperitoneal incision was made and then the distal renal nerve was cut to eliminate its afferent activity. The nerve signals were amplified with a high pass filter at 10 Hz and a low pass filter at 3000 Hz by a four-channel AC/DC differential amplifier (DP-304, Warner Instruments, Hamden, CT) and the RSNA was integrated at a time constant of 100 ms. At the end of each experiment, the background noise was determined after section of the central end of the nerve and was subtracted from the integrated values of the RSNA.[36] The raw and integrated RSNA, ABP, MAP, and HR were recorded by a PowerLab data acquisition system (8/35, AD Instruments).

Evaluation of CSAR

To expose the heart, a limited left lateral thoracotomy was performed and then the pericardium was removed. The CSAR was induced by stimulating cardiac sympathetic afferents with a piece of filter paper (3 mm × 3 mm) containing capsaicin (1.0 nmol in 2.0 μL) on the anterior wall of the left ventricle. The filter paper was removed 1 min later, and the ventricular surface was rinsed three times with normal saline (10 mL) at 37°C. The CSAR was evaluated by the RSNA and MAP responses to the epicardial application of capsaicin.[36] The RSNA was expressed as the percent change from the baseline value. RSNA change (%) = $(RSNA_{before} - RSNA_{after})/RSNA_{before} \times 100$.

PVN microinjection

According to Paxinos & Watson's rat atlas, the stereotaxic coordinates using stoelting (Chicago, USA) for the PVN are 1.8 mm caudal from bregma, 0.4 mm lateral to the midline and 7.9 mm ventral to the dorsal surface. The microinjection volume was 50 nL for each side of the PVN and the bilateral PVN microinjections were completed within 1 min. At the end of the experiment, 2% Evans Blue dye (50 nL) was injected into each site and then the microinjection site was verified with microscope histologically. The rats were

excluded from data analysis for the microinjection sites were outside the PVN.

Chemicals

Rat IMD, CGRP receptor antagonist CGRP8-37 and AM receptor antagonist AM22-52 were from Bachem AG (Hauptstrasse, Bubendorf, Switzerland); non-selective NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME), $GABA_A$ receptor antagonist gabazine and $GABA_B$ receptor antagonist CGP-35348 were purchased from Sigma Chemical Co (St. Louis, MO). These chemicals were dissolved in saline.

Experiment protocol

Experiment 1. Rats were first randomly divided into nine groups ($n = 6$ for each group), which were subjected to bilateral PVN microinjection of saline, three doses of IMD (0.5, 5 and 25 pmol), CGRP receptor antagonist CGRP8-37 (0.2 nmol), AM receptor antagonist AM22-52 (1 nmol), non-selective NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME) (200 nmol), $GABA_A$ receptor antagonist gabazine (0.1 nmol) or $GABA_B$ receptor antagonist CGP-35348 (10 nmol) in Sham rats and 2K1C rats. The CSAR was induced 10 min after saline, CGRP8-37, AM22-52, L-NAME, gabazine or CGP-35348 microinjection into the PVN and 30 min after IMD microinjection into the PVN. The changes of RSNA and MAP responses to capsaicin were determined by averaging 30 s of the maximal responses ($n = 6$ for each group). To exclude the possibility that the effect of IMD on the CSAR was caused by diffusion to other brain area, the effect of microinjection of IMD (25 pmol) into the anterior hypothalamic area which is adjacent to the PVN was determined in 2K1C rats ($n = 3$).

Experiment 2. Effects of PVN pretreatment with CGRP8-37 (0.2 nmol), AM22-52 (1 nmol), L-NAME (200 nmol), gabazine (0.1 nmol) and CGP-35348 (10 nmol) on the CSAR response to the PVN microinjection of IMD (25 pmol) were investigated, respectively, in five groups of Sham rats and five groups of 2K1C rats ($n = 6$ for each group). The pretreatment was performed 10 min before IMD treatment in the PVN, and the CSAR was induced 30 min after IMD.

Statistical analysis

The RSNA and MAP responses to epicardial application of capsaicin were determined by averaging 30 s of the parameters beginning at the 16th second after epicardial application of capsaicin. The RSNA change was expressed as the percent change from the baseline values. All data were expressed as mean ± SE. One-way analysis of variance (ANOVA), followed by *post hoc* Bonferroni test, was used when multiple comparisons were made. Comparisons between two groups were assessed by Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

General data

There was no significant difference in the body weight (BW) and baseline HR between Sham and 2K1C groups, but SBP, LVW, LVW/BW and MAP in 2K1C rats were significantly higher than those in Sham rats (Table 1).

Effects of each chemical on the baseline RSNA and MAP

Bilateral PVN microinjection of IMD (25 pmol) caused greater decreases in the baseline RSNA and MAP in 2K1C rats than in Sham rats, but it had not significant effect on HR (data not shown). PVN microinjection of AM22-52 (in 2K1C rats), L-NAME, gabazine or CGP-35348 (in both Sham rats and 2K1C rats) significantly increased the baseline RSNA and MAP, but CGRP receptor antagonist CGRP8-37 had no significant effects on them in Sham and 2K1C rats (Table 2).

Effects of different doses of IMD on the CSAR

Microinjection of three doses of IMD (0.5, 5 and 25 pmol) into the PVN caused dose-related decreases in the CSAR by evaluating the RSNA and MAP responses to the epicardial application of capsaicin in both Sham rats and 2K1C rats. The effect of IMD on CSAR in 2K1C rats was greater than that in Sham rats (Figure 1). Representative recordings showed that PVN microinjection of IMD (25 pmol) attenuated the CSAR in 2K1C rat (Figure 2b). In addition, microinjection of IMD (25 pmol) into the anterior hypothalamic area had no significant effect on the CSAR (data not shown).

Effects of CGRP8-37 and AM22-52 on the CSAR

Microinjection of CGRP receptor antagonist CGRP8-37 into the PVN had no significant effect on the CSAR in both Sham and 2K1C rats. However, AM receptor antagonist AM22-52 significantly increased the CSAR in Sham and 2K1C rats (Figure 3). The CSAR-inhibitory effect of IMD was abolished by the pretreatment with AM22-52 but not CGRP8-37 in the PVN in both Sham rats and 2K1C rats (Figure 3).

Table 1 General characteristics of rats

	Sham	2K1C
Body weight (g)	323 ± 19	319 ± 18
Left ventricular weight (mg)	520 ± 27	735 ± 29*
LVW/BW ratio (mg g^{-1})	1.61 ± 0.06	2.30 ± 0.09*
SBP (mmHg)	121 ± 6	198 ± 9*
Baseline MAP (mmHg)	91 ± 5	140 ± 7*
Baseline HR (beats/min)	364 ± 25	381 ± 28

Sham: sham operated; 2K1C: 2-kidney 1-clip; LVW: left ventricular weight; BW: body weight; SBP: systolic blood pressure, which was measured in conscious state by a noninvasive computerized tail-cuff system; MAP: mean arterial pressure and HR: heart rate. Baseline MAP and HR were measured under anesthesia with a pressure transducer through a catheter in the right carotid artery. Values are mean ± SE.

*$P < 0.05$ compared with the Sham. $n = 7$ for each group.

Table 2 Changes of the baseline RSNA and MAP caused by PVN microinjection of saline, IMD (25 pmol), CGRP8-37 (0.2 nmol), AM22-52 (1 nmol), L-NAME (200 nmol), gabazine (0.1 nmol) or CGP-35348 (10 nmol) in Sham and 2K1C rats

Groups	Sham RSNA change (%)	MAP change (mmHg)	2K1C RSNA change (%)	MAP change M (mmHg)
Saline	−0.43 ± 1.07	−0.93 ± 0.84	0.68 ± 0.76	0.18 ± 0.60
IMD	−8.16 ± 1.24*	−3.73 ± 1.57*	−19.86 ± 1.52*#	−17.95 ± 1.56*#
CGRP8-37	0.74 ± 1.83	1.15 ± 1.29	1.18 ± 1.19	1.21 ± 1.16
AM22-52	2.39 ± 0.68	2.85 ± 0.30	6.82 ± 0.66*#	6.59 ± 0.95*#
L-NAME	10.66 ± 1.29*	9.46 ± 1.94*	7.33 ± 1.05*	5.00 ± 1.81*#
Gabazine	22.67 ± 3.36*	21.61 ± 2.14*	20.20 ± 2.39*	19.84 ± 2.15*
CGP-35348	12.27 ± 1.90*	4.85 ± 0.95*	13.61 ± 2.12*	5.34 ± 0.82*

Sham: sham operated; 2K1C: 2-kidney 1-clip; RSNA: renal sympathetic nerve activity; MAP: mean arterial pressure. Values are mean ± SE.
*$P < 0.05$ compared with saline. $n = 6$ for each group.
#$P < 0.05$ compared with Sham. $n = 6$ for each group.

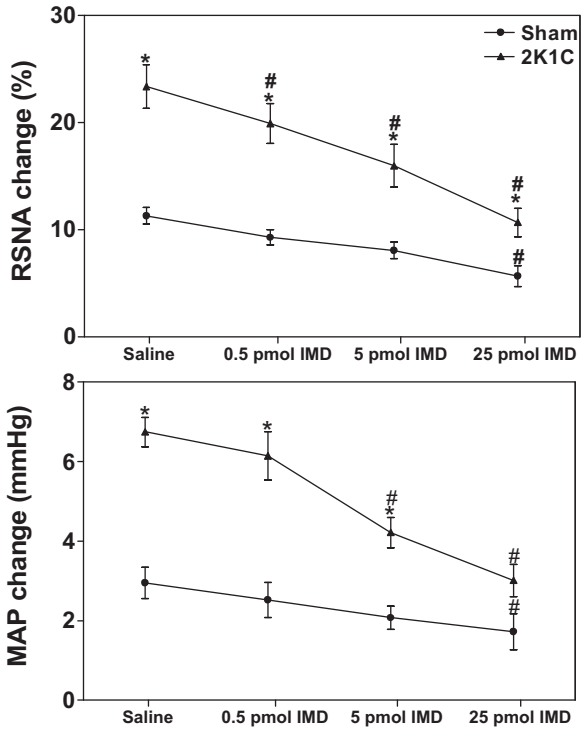

Figure 1 Effect of PVN microinjection of saline and three doses of IMD (0.5, 5 or 25 pmol) on the CSAR in sham-operated (Sham) and 2-kidney-1-clip (2K1C) rats. The CSAR induced by epicardial application of capsaicin (1 nmol) was evaluated by the RSNA and MAP responses to capsaicin. CSAR: cardiac sympathetic afferent reflex; RSNA: renal sympathetic nerve activity; MAP: mean arterial pressure. Values are mean ± SE. *$P < 0.05$ *vs*. Sham; #$P < 0.05$ *vs*. saline. $n = 6$ for each group

Effects of L-NAME on the CSAR

Non-selective NOS inhibitor L-NAME application into the PVN significantly increased the CSAR in both Sham rats and 2K1C rats (Figure 4). Pretreatment with L-NAME in the PVN prevented the effect of IMD on CSAR in both Sham rat and 2K1C rat (Figure 4).

Effects of gabazine and CGP-35348

Microinjection of $GABA_B$ receptor antagonist into the PVN significantly increased the CSAR in both Sham rats and 2K1C rats but not $GABA_A$ receptor antagonist gabazine (Figure 5). In both Sham rats and 2K1C rats, the CSAR-inhibitory effect of IMD was attenuated by the PVN pretreatment with CGP-35348 but not gabazine (Figure 5).

Discussion

Our previous studies have indicated that CSAR is enhanced in rats with hypertension.[20,21] NO and GABA have been shown to be involved in the regulation of CSAR in rats.[34,37] IMD in the PVN inhibits sympathetic overdrive and attenuates hypertension in 2K1C rats, which are mediated by AM receptors.[38] We have now shown that IMD in the PVN decreases the enhanced CSAR and AM22-52 prevents the effect of IMD on CSAR in 2K1C rats. Pretreatment with non-selective NOS inhibitor L-NAME and $GABA_B$ receptor antagonist CGP-35348 significantly attenuated the CSAR response to IMD but not $GABA_A$ receptor antagonist gabazine in the PVN in both Sham and 2K1C rats. These results suggest that IMD in the PVN inhibits the CSAR via AM receptor, NO and GABA in the PVN involve in the inhibitory effect of IMD on CSAR in normal and renovascular hypertensive rats.

IMD sits within the CGRP/CT family of peptides, which includes calcitonin, CGRP, amylin and AM.[2,4] In the brain, IMD involves in controlling the homeostasis of growth and metabolism. For instance, IMD stimulates prolactin, oxytocin and vasopressin release,[28,39,40] induces anti-diuresis and anti-natriuresis, suppresses food and water intake,[5] growth hormone release and gastric emptying activity.[2] Intracerebroventricular administration of IMD causes an increase in BP,[5] which is opposite to its peripheral effect. These actions suggest that IMD is potentially an important regulatory factor in cardiovascular activities via central and peripheral actions. It is interesting that AM has different effects on BP depending on where it is applied in the brain.[34,41] AM administration into the PVN decreases BP,[34] whereas in the lateral ventricle or NTS, AM increases BP.[42,43] Similar to AM, IMD microinjection into the lateral ventricle and NTS can cause an increase in BP,[5,7,44] but PVN microinjection of IMD decreases RSNA and BP in normal and renovascular hypertensive rats.[38] These results indicate that IMD may have effect on the CSAR in the PVN. In the present study, IMD in the PVN significantly inhibited the

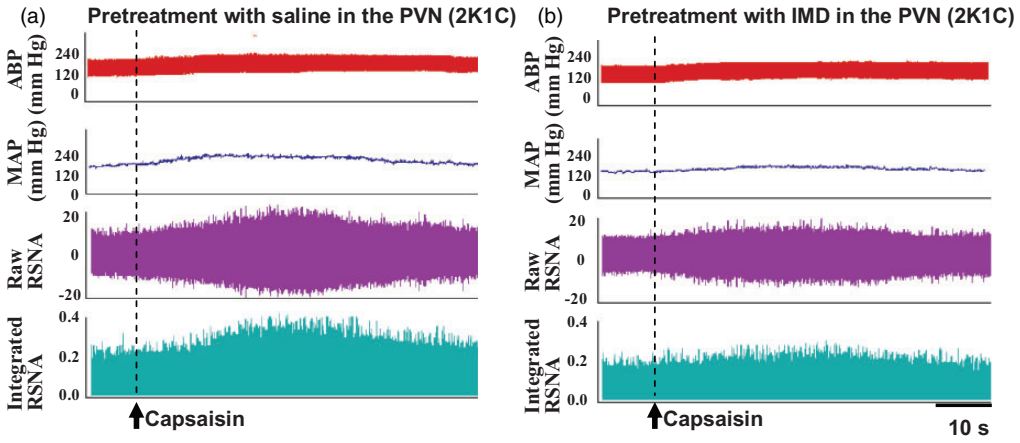

Figure 2 Tracing showing the effect of microinjection of IMD (25 pmol) into the PVN on CSAR in 2-kidney-1-clip (2K1C) rats. The CSAR induced by epicardial application of capsaicin (1 nmol) was evaluated by the RSNA and MAP responses to capsaicin. The enhanced CSAR in 2K1C rats (a) was attenuated by IMD in the PVN (b). CSAR: cardiac sympathetic afferent reflex; RSNA: renal sympathetic nerve activity; ABP: arterial blood pressure; MAP: mean arterial pressure. (A color version of this figure is available in the online journal.)

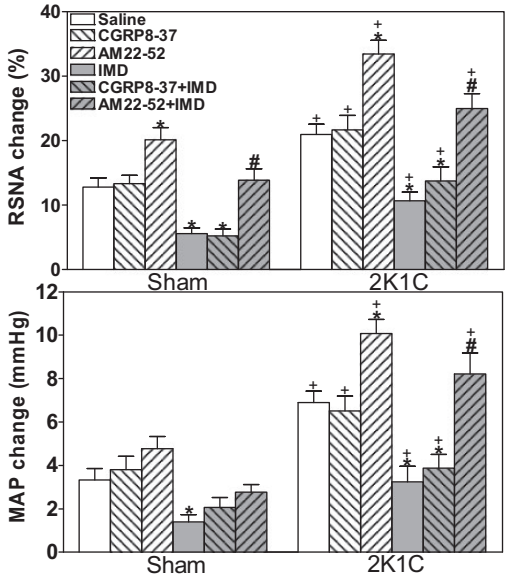

Figure 3 The effect of PVN microinjection of saline, CGRP receptor antagonist CGRP8-37 (0.2 nmol), AM receptor antagonist AM22-52 (1 nmol) or IMD (25 pmol) on CSAR and the effect of PVN microinjection of IMD on CSAR after pretreatment with CGRP8-37 or AM22-52 in both Sham rats and 2-kidney-1-clip (2K1C) rats. IMD was administered 10 min after the microinjection of CGRP8-37 or AM22-52. Values are mean ± SE. $^{*}P < 0.05$ compared with saline; $^{\#}P < 0.05$ compared with IMD; $^{+}P < 0.05$ compared with Sham. $n = 6$ for each group

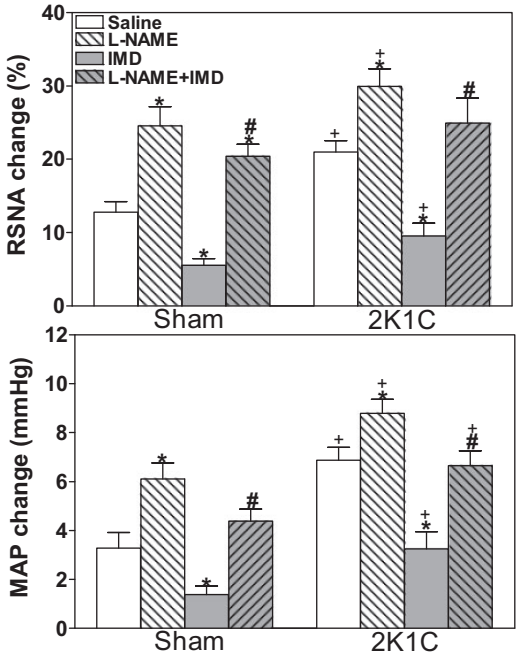

Figure 4 The effect of PVN microinjection of saline, IMD (25 pmol) or L-NAME (200 nmol) on CSAR and the effect of PVN microinjection of IMD on CSAR after pretreatment with L-NAME in both Sham rats and 2-kidney-1-clip (2K1C) rats. IMD was administered 10 min after the microinjection of L-NAME. Values are mean ± SE. $^{*}P < 0.05$ compared with saline; $^{\#}P < 0.05$ compared with IMD; $^{+}P < 0.05$ compared with Sham. $n = 6$ for each group

enhanced CSAR and the baseline RSNA and MAP in 2K1C rats, which suggests that the inhibition of CSAR by IMD in the PVN is an important mechanism involved in the inhibitory effects of IMD on sympathetic nerve activity in renovascular hypertension.

In the present, no unique receptor has been identified for IMD which shares the receptor system consisting of CRLR and RAMPs with CGRP and AM. The CRLR/RAMP1 complex forms the CGRP receptor, CRLR/RAMP2 or CRLR/RAMP3 forms the AM receptor, IMD can non-selectively bind to the three CRLR/RAMP complexes.[2] In this study, IMD microinjection into the PVN caused greater decrease in the CSAR, but it did not affect the CSAR outside the PVN microinjection, which suggests that the effect of IMD on CSAR is specific for the PVN. The inhibitory of IMD on CSAR was stronger in 2K1C rats than in Sham rats. PVN AM receptor antagonist AM22-52 increased the CSAR and

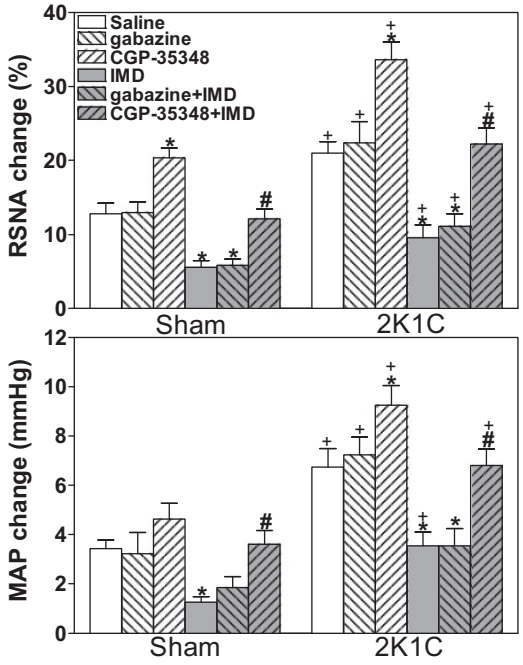

Figure 5 The effect of PVN microinjection of saline, IMD (25 pmol), gabazine (0.1 nmol) or CGP-35348 (10 nmol) on CSAR and the effect of PVN microinjection of IMD on CSAR after pretreatment with gabazine or CGP-35348 in both Sham rats and 2-kidney-1-clip (2K1C) rats. IMD was administered 10 min after the microinjection gabazine or CGP-35348. Values are mean ± SE. $^{*}P < 0.05$ compared with saline; $^{\#}P < 0.05$ compared with IMD; $^{+}P < 0.05$ compared with Sham. $n = 6$ for each group

its pretreatment in the PVN attenuated the effects of IMD on CSAR in both Sham and 2K1C rats. However, CGRP receptor antagonist CGRP8-37 had no significant effects. These results indicate that exogenous IMD in the PVN inhibits the CSAR partially via AM receptor rather than CGRP receptor. Activation of the AM receptor by endogenous ligands in the PVN is involved in the regulation of CSAR in physiological state, and plays a tonic role in attenuating the CSAR in renovascular hypertensive rats. Furthermore, PVN CRLR, RAMP2 and RAMP3 protein expressions are significantly increased, but IMD is decreased in 2K1C rats,[38] which suggests that the AM receptor (CRLR/RAMP2 or CRLR/RAMP3) rather than CGRP receptors (CRLR/RAMP1) are mainly involved in the enhanced CSAR in 2K1C rats. The higher AM receptor expression and lower IMD expression in the PVN may be important in the regulation of CSAR in renal hypertensive rats.

NO is an atypical neurotransmitter that elicits inhibitory effects on cardiovascular function. In the PVN, NO inhibits the RSNA response to angiotensin II and its level is downregulated in renal hypertension.[30,45] NO mediates the effects of IMD in the PVN on the RSNA and MAP in 2K1C rats and peripheral vasodilatation.[6,28,29,38] In this study, our results showed that non-selective NOS inhibitor L-NAME prevented IMD-induced decrease in the CSAR and IMD microinjection into the PVN significantly increased NO content in 2K1C,[38] which indicates that IMD may activate NO-producing neurons and increase NO production in the PVN, and that NO, in turn, decreases the CSAR.

GABA exerts a tonic inhibitory effect on the sympathetic nervous system at the level of the PVN,[31,32] the GABAergic activity is increased because of the result of an increase in GABA release in the PVN in chronic renal-wrap hypertension. Selective $GABA_A$ receptor antagonist, bicuculline, abolishes AM-induced hyperpolarization of magnocellular neurons and hypotensive effect in the PVN.[34,46] Moreover, endogenous GABA in the PVN has an important role in regulating the CSAR.[33] In the present study, the effect of IMD on CSAR was attenuated by $GABA_B$ receptor blocker CGP-35348 not $GABA_A$ receptor blocker gabazine, and CGP-35348 strengthened the CSAR in both Sham rats and 2K1C rats, while gabazine had no significant effect on the CSAR. These results indicate that PVN GABA involved in the effect of IMD on CSAR in both Sham rats and 2K1C rats, and $GABA_B$ receptor in the PVN could mediate the effect of GABA on CSAR in renal hypertensive rats.

PVN integrates signals from a wide variety of neurons, these include presynaptic neurons projecting to the PVN from other important integrative centers including subfornical organ, arcuate nucleus, NTS and so on. Neurons originating within the PVN project to rostral ventrolateral medulla and intermediolateral column of spinal cord. The molecular and neuroanatomical mechanisms by which IMD acts on the neural circuits within the autonomic system remain unclear. In this study, the action of PVN microinjection of IMD on CSAR is mediated by the AM receptor CRLR/RAMP2 or CRLR/RAMP3. Alternatively, it is possible that IMD interacts with an as yet unidentified receptor. In the rat brain, the abundant IMD-like immunopositive cells were distributed in PVN including both parvocellular and magnocellular neurons. It is possible that IMD induces suppression of CSAR mainly by the activation of NO and GABAergic neurons via AM receptor or unidentified receptor, all leading to the attenuation of CSAR, and resulting in the decrease in sympathetic outflow. Indeed, we found that the effects of IMD microinjection into the PVN were attenuated by pretreatment of NOS inhibitor or GABA receptor antagonist. To truly demonstrate the important role of IMD in pathophysiology of hypertension, it will be necessary to create animal models by embryonic knockout, antisense or small interfering RNA, etc. If so, IMD's production or action has been involved.

The sympathoinhibitory actions of NO are mediated by GABA within the PVN.[47–49] IMD in the PVN may first activate NO-producing cells to increase the production and release of NO which stimulates GABAergic neurons or IMD may simultaneously activate NO-producing cells and GABAergic neurons, which needs us to further explore in future study.

In conclusion, IMD in the PVN effectively attenuated the CSAR in the PVN in 2K1C rats. The PVN is a key site for the IMD-induced inhibition of CSAR. The CSAR-inhibitory role of IMD may be an important mechanism for the decrease of excessive sympathetic activation in 2K1C rats. Therefore, long-term application of IMD in the PVN may effectively attenuate the excessive sympathetic activation by inhibiting the enhanced CSAR, thereby decrease blood pressure, reduce pressure load on the heart and improves myocardial

remodeling and ventricular dysfunction in renovascular hypertension.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. Y-bZ and G-qZ designed the study and wrote the paper; HZ, H-jS and J-rC performed the research; LD and QG analyzed the data and C-sT provided the chemicals.

ACKNOWLEDGEMENTS

This work was supported by the funds from the National Natural Science Foundation of China (grant no. 81000106); the Jiangsu Higher Education Institutions of China (grant no. 10KJB310004) and the Nanjing Medical University of China (grant no. 09JC013, JX 10131801056, 09NJMUZ04 and 2012NJMU025).

REFERENCES

1. Hong Y, Hay DL, Quirion R, Poyner DR. The pharmacology of adrenomedullin 2/intermedin. *Br J Pharmacol* 2012;**166**:110–20
2. Roh J, Chang CL, Bhalla A, Klein C, Hsu SY. Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. *J Biol Chem* 2004;**279**:7264–74
3. Takahashi K, Kikuchi K, Maruyama Y, Urabe T, Nakajima K, Sasano H, Imai Y, Murakami O, Totsune K. Immunocytochemical localization of adrenomedullin 2/intermedin-like immunoreactivity in human hypothalamus, heart and kidney. *Peptides* 2006;**27**:1383–9
4. Takahashi K, Morimoto R, Hirose T, Satoh F, Totsune K. Adrenomedullin 2/intermedin in the hypothalamo-pituitary-adrenal axis. *J Mol Neurosci* 2011;**43**:182–92
5. Taylor MM, Bagley SL, Samson WK. Intermedin/adrenomedullin-2 acts within central nervous system to elevate blood pressure and inhibit food and water intake. *Am J Physiol Regul Integr Comp Physiol* 2005;**288**:R919–27
6. Jolly L, March JE, Kemp PA, Bennett T, Gardiner SM. Mechanisms involved in the regional haemodynamic effects of intermedin (adrenomedullin 2) compared with adrenomedullin in conscious rats. *Br J Pharmacol* 2009;**157**:1502–13
7. Ren YS, Yang JH, Zhang J, Pan CS, Yang J, Zhao J, Pang YZ, Tang CS, Qi YF. Intermedin 1-53 in central nervous system elevates arterial blood pressure in rats. *Peptides* 2006;**27**:74–9
8. Biaggioni I. Should we target the sympathetic nervous system in the treatment of obesity-associated hypertension? *Hypertension* 2008;**51**:168–71
9. Han Y, Yuan N, Zhang SJ, Gao J, Shi Z, Zhou YB, Gao XY, Zhu GQ. c-Src in paraventricular nucleus modulates sympathetic activity and cardiac sympathetic afferent reflex in renovascular hypertensive rats. *Pflugers Arch* 2011;**461**:437–46
10. Hogarth AJ, Mackintosh AF, Mary DA. The effect of gender on the sympathetic nerve hyperactivity of essential hypertension. *J Hum Hypertens* 2007;**21**:239–45
11. Li P, Sun HJ, Cui BP, Zhou YB, Han Y. Angiotensin-(1-7) in the rostral ventrolateral medulla modulates enhanced cardiac sympathetic afferent reflex and sympathetic activation in renovascular hypertensive rats. *Hypertension* 2013;**61**:820–7
12. Rubinger D, Backenroth R, Sapoznikov D. Sympathetic activation and baroreflex function during intradialytic hypertensive episodes. *PLoS One* 2012;**7**:e36943
13. Mancia G, Grassi G, Giannattasio C, Seravalle G. Sympathetic activation in the pathogenesis of hypertension and progression of organ damage. *Hypertension* 1999;**34**:724–8
14. Morise T, Horita M, Kitagawa I, Shinzato R, Hoshiba Y, Masuya H, Suzuki M, Takekoshi N. The potent role of increased sympathetic tone in pathogenesis of essential hypertension with neurovascular compression. *J Hum Hypertens* 2000;**14**:807–11
15. Rahn KH, Barenbrock M, Hausberg M. The sympathetic nervous system in the pathogenesis of hypertension. *J Hypertens Suppl* 1999;**17**:S11–4
16. Del Colle S, Morello F, Rabbia F, Milan A, Naso D, Puglisi E, Mulatero P, Veglio F. Antihypertensive drugs and the sympathetic nervous system. *J Cardiovasc Pharmacol* 2007;**50**:487–96
17. Fisher JP, Fadel PJ. Therapeutic strategies for targeting excessive central sympathetic activation in human hypertension. *Exp Physiol* 2010;**95**:572–80
18. Grassi G, Seravalle G, Quarti-Trevano F. The 'neuroadrenergic hypothesis' in hypertension: current evidence. *Exp Physiol* 2010;**95**:581–6
19. Malliani A, Montano N. Emerging excitatory role of cardiovascular sympathetic afferents in pathophysiological conditions. *Hypertension* 2002;**39**:63–8
20. Zhu GQ, Xu Y, Zhou LM, Li YH, Fan LM, Wang W, Gao XY, Chen Q. Enhanced cardiac sympathetic afferent reflex involved in sympathetic overactivity in renovascular hypertensive rats. *Exp Physiol* 2009;**94**:785–94
21. Yuan N, Zhang F, Zhang LL, Gao J, Zhou YB, Han Y, Zhu GQ. SOD1 gene transfer into paraventricular nucleus attenuates hypertension and sympathetic activity in spontaneously hypertensive rats. *Pflugers Arch* 2013;**465**:261–70
22. Han Y, Sun HJ, Li P, Gao Q, Zhou YB, Zhang F, Gao XY, Zhu GQ. Angiotensin-(1-7) in paraventricular nucleus modulates sympathetic activity and cardiac sympathetic afferent reflex in renovascular hypertensive rats. *PLoS One* 2012;**7**:e48966
23. Zhong MK, Duan YC, Chen AD, Xu B, Gao XY, De W, Zhu GQ. Paraventricular nucleus is involved in the central pathway of cardiac sympathetic afferent reflex in rats. *Exp Physiol* 2008;**93**:746–53
24. Hashimoto H, Kitamura K, Kawasaki M, Saito T, Suzuki H, Otsubo H, Ohbuchi T, Yokoyama T, Fujihara H, Takei Y, Ueta Y. Adrenomedullin 2/intermedin-like immunoreactivity in the hypothalamus and brainstem of rats. *Auton Neurosci* 2008;**139**:46–54
25. Chan SH, Chan JY. Brain stem NOS and ROS in neural mechanisms of hypertension. *Antioxid Redox Signal*. Epub ahead of print 2013
26. Gamboa A, Okamoto LE, Diedrich A, Choi L, Robertson D, Farley G, Paranjape S, Biaqqioni L. Sympathetic activation and nitric oxide function in early hypertension. *Am J Physiol Heart Circ Physiol* 2012;**302**:H1438–43
27. Shinohara K, Hirooka Y, Kishi T, Sunagawa K. Reduction of nitric oxide-mediated γ-amino butyric acid release in rostral ventrolateral medulla is involved in superoxide-induced sympathoexcitation of hypertensive rats. *Circ J* 2012;**76**:2814–21
28. Yang JH, Pan CS, Jia YX, Zhang J, Zhao J, Pang YZ, Yang CS, Qi YF. Intermedin1–53 activates L-arginine/nitric oxide synthase/nitric oxide pathway in rat aortas. *Biochem Biophys Res Commun* 2006;**341**:567–72
29. Kandilci HB, Gumusel B, Lippton H. Intermedin/adrenomedullin-2 (IMD/AM2) relaxes rat main pulmonary arterial rings via cGMP-dependent pathway: role of nitric oxide and large conductance calcium-activated potassium channels (BK(Ca)). *Peptides* 2008;**29**:1321–28
30. Northcott CA, Billecke S, Craig T, Hinojosa-Laborde C, Patel KP, Chen AF, D'Alecy LG, Haywood JR. Nitric oxide synthase, ADMA, SDMA, and nitric oxide activity in the paraventricular nucleus throughout the etiology of renal wrap hypertension. *Am J Physiol Heart Circ Physiol* 2012;**302**:H2276–84
31. Chen QH, Haywood JR, Toney GM. Sympathoexcitation by pvn-injected bicuculline requires activation of excitatory amino acid receptors. *Hypertension* 2003;**42**:725–31
32. Martin DS, Segura T, Haywood JR. Cardiovascular responses to bicuculline in the paraventricular nucleus of the rat. *Hypertension* 1991;**18**:48–55
33. Zhong MK, Shi Z, Zhou LM, Gao J, Liao ZH, Wang W, Gao XY, Zhu GQ. Regulation of cardiac sympathetic afferent reflex by GABA(A) and

GABA(B) receptors in paraventricular nucleus in rats. *Eur J Neurosci* 2008;**27**:3226–32
34. Xu Y, Krukoff TL. Decrease in arterial pressure induced by adrenomedullin in the hypothalamic paraventricular nucleus is mediated by nitric oxide and GABA. *Regul Pept* 2004;**119**:21–30
35. Chen AD, Zhang SJ, Yuan N, Xu Y, De W, Gao XY, Zhu GQ. AT1 receptors in paraventricular nucleus contribute to sympathetic activation and enhanced cardiac sympathetic afferent reflex in renovascular hypertensive rats. *Exp Physiol* 2011;**96**:94–103
36. Zhang L, Xiong XQ, Fan ZD, Gan XB, Gao XY, Zhu GQ. Involvement of enhanced cardiac sympathetic afferent reflex in sympathetic activation in early stage of diabetes. *J Appl Physiol* 2012;**113**:47–55
37. Zhu GQ, Gao XY, Zhang F, Wang W. Reduced nitric oxide in the rostral ventrolateral medulla enhances cardiac sympathetic afferent reflex in rats with chronic heart failure. *Sheng Li Xue Bao* 2004;**56**:47–53
38. Zhou YB, Sun HJ, Chen D, Liu TY, Han Y, Wang JJ, Tang CS, Kang YM, Zhu GQ. Intermedin in paraventricular nucleus attenuates sympathetic activity and blood pressure via nitric oxide in hypertensive rats. *Hypertension* 2014;**63**:330–7
39. Lin Chang C, Roh J, Park JI, Klein C, Cushman N, Haberberger RV, Hsu SY. Intermedin functions as a pituitary paracrine factor regulating prolactin release. *Mol Endocrinol* 2005;**19**:2824–38
40. Taylor MM, Samson WK. Stress hormone secretion is altered by central administration of intermedin/adrenomedullin-2. *Brain Res* 2005;**1045**:199–205
41. Cam-Etoz B, Isbil-Buyukcoskun N, Ozluk K. Cardiovascular effects of the intracerebroventricular injection of adrenomedullin: roles of the peripheral vasopressin and central cholinergic systems. *Braz J Med Biol Res* 2012;**45**:250–5
42. Bell D, McDermott BJ. Intermedin (adrenomedullin-2): a novel counter-regulatory peptide in the cardiovascular and renal systems. *Br J Pharmacol* 2008;**153**(Suppl 1): S247–62
43. Yen DH, Chen LC, Shen YC, Chiu YC, Ho IC, Lou YJ, Chen IC, Yen JC. Protein kinase A-dependent neuronal nitric oxide synthase activation mediates the enhancement of baroreflex response by adrenomedullin in the nucleus tractus solitarii of rats. *J Biomed Sci* 2011;**18**:32
44. Li P, Sun HJ, Han Y, Wang JJ, Zhang F, Tang CS, Zhou YB. Intermedin enhances sympathetic outflow via receptor-mediated cAMP/PKA signaling pathway in nucleus tractus solitarii of rats. *Peptides* 2013;**47**:1–6
45. Li YF, Wang W, Mayhan WG, Patel KP. Angiotensin-mediated increase in enal sympathetic nerve discharge within the PVN: role of nitric oxide. *Am J Physiol Regul Integr Comp Physiol* 2006;**290**:R1035–43
46. Follwell MJ, Ferguson AV. Adrenomedullin influences magnocellular and parvocellular neurons of paraventricular nucleus via separate mechanisms. *Am J Physiol Regul Integr Comp Physiol* 2002;**283**:R1293–302
47. Hsu YC, Chen HI, Kuo YM, Yu L, Huang TY, Chen SJ, Chuang JI, Wu FS, Jen CJ. Chronic treadmill running in normotensive rats resets the resting blood pressure to lower levels by upregulating the hypothalamic GABAergic system. *J Hypertens* 2011;**29**:2339–48
48. Li DP, Chen SR, Pan HL. Nitric oxide inhibits spinally projecting paraventricular neurons through potentiation of presynaptic GABA release. *J Neurophysiol* 2002;**88**:2664–74
49. Zhang K, Patel KP. Effect of nitric oxide within the paraventricular nucleus on renal sympathetic nerve discharge: role of GABA. *Am J Physiol* 1998;**275**:R728–34

(Received December 26, 2013, Accepted March 10, 2014)