Intermedin in Paraventricular Nucleus Attenuates Sympathetic Activity and Blood Pressure via Nitric Oxide in Hypertensive Rats

Ye-Bo Zhou, Hai-Jian Sun, Dan Chen, Tong-Yan Liu, Ying Han, Jue-Jin Wang, Chao-Shu Tang, Yu-Ming Kang, Guo-Qing Zhu

Abstract—Intermedin (IMD) is a member of calcitonin/calcitonin gene–related peptide family, which shares the receptor system consisting of calcitonin receptor–like receptor (CRLR) and receptor activity–modifying proteins (RAMPs). This study investigated the effects of IMD in paraventricular nucleus (PVN) on renal sympathetic nerve activity and mean arterial pressure and its downstream mechanism in hypertension. Rats were subjected to 2-kidney 1-clip (2K1C) surgery to induce renovascular hypertension or sham operation. Acute experiments were performed 4 weeks later under anesthesia. IMD mRNA and protein were downregulated in 2K1C rats. Bilateral PVN microinjection of IMD caused greater decreases in renal sympathetic nerve activity and mean arterial pressure in 2K1C rats than in sham-operated rats, which were prevented by pretreatment with adrenomedullin receptor antagonist AM22-52 or nonselective nitric oxide (NO) synthase inhibitor NG-nitro-L-arginine methyl ester, and attenuated by selective neuronal NO synthase inhibitor Nω-propyl-l-arginine hydrochloride or endothelial NO synthase inhibitor N(5)-(1-iminoethyl)-l-ornithine dihydrochloride. AM22-52 increased renal sympathetic nerve activity and mean arterial pressure in 2K1C rats but not in sham-operated rats, whereas calcitonin/calcitonin gene–related peptide receptor antagonist calcitonin/calcitonin gene–related peptide 8-37 had no significant effect. CRLR and RAMP3 mRNA, as well as CRLR, RAMP2, and RAMP3 protein expressions, in the PVN were increased in 2K1C rats. Microinjection of IMD into the PVN increased the NO metabolites (NOx) level in the PVN in 2K1C rats, which was prevented by AM22-52. Chronic PVN infusion of IMD reduced, but AM22-52 increased, blood pressure in conscious 2K1C rats. These results indicate that IMD in the PVN inhibits sympathetic activity and attenuates hypertension in 2K1C rats, which are mediated by adrenomedullin receptors (CRLR/RAMP2 or CRLR/RAMP3) and its downstream NO. (Hypertension. 2014;63:330-337.) ● Online Data Supplement

Key Words: hypertension ■ intermedin protein, rat ■ nitric oxide ■ paraventricular hypothalamic nucleus

Intermedin (IMD) or adrenomedullin (AM) 2 is a member of the calcitonin/calcitonin gene–related peptide (CGRP) family discovered in 2004.1 Unlike other members of this family, no unique receptor has yet been identified for IMD. IMD, CGRP, and AM share the receptor system consisting of calcitonin receptor–like receptor (CRLR) and receptor activity–modifying proteins (RAMPs). The CRLR/RAMP3 forms the CGRP receptor, whereas CRLR/RAMP2 or CRLR/RAMP3 complex forms the AM receptor. IMD binds nonselectively to all 3 CRLR/RAMP complexes.2 IMD is widely distributed in peripheral organs and the central nervous system.3,4 CRLR and RAMPs have been found in the paraventricular nucleus (PVN) of hypothalamus,5,6 and abundant IMD-like immunoreactivity have been found in the PVN including parvocellular and magnocellular cells.6,7 PVN is an important integrative site implicated in the neurogenic component of 2-kidney 1-clip (2K1C)–induced renovascular hypertension8 and contributes to sympathetic activation and hypertension in 2K1C rats.9 Intracerebroventricular injection of IMD increased mean arterial pressure (MAP) and sympathetic activity.10 A recent study in our laboratory has shown that microinjection of IMD into the nucleus tractus solitarii (NTS) increased renal sympathetic nerve activity (RSNA) and MAP.11 It is interesting to know the roles and mechanisms of IMD in the PVN in sympathetic activation and hypertension because of the importance of the PVN in the pathogenesis of hypertension in 2K1C rats.

Inhibition of nitric oxide (NO) synthase (NOS) in the PVN increased MAP and heart rate (HR), whereas NO donor sodium nitroprusside produced depressor responses in normal rats.12 Downregulation of NO was involved in renal wrap
hypertension. It has been found that IMD1-53 increases NO content and NOS activity in isolated rat aortas, and inhibition of NOS is more effective in reducing the vasodilator effects of IMD than that of AM. It is not known whether NO in the PVN is involved in the effects of IMD. The present study was designed to determine whether IMD in the PVN attenuates sympathetic activation and hypertension and whether the receptor CRLR/RAMPs and NO in the PVN are involved in the effects of IMD in renovascular hypertensive rats. Furthermore, the integration of the effects of IMD in NTS and PVN was investigated.

Methods

Experiments were performed in male Sprague–Dawley rats. 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 rats were housed in a temperature-controlled and humidity-controlled room with a 12:12-hour light–dark cycle with standard chow and tap water ad libitum.

Renovascular hypertension was induced by the 2K1C method in male rats weighing 160 to 180 g, as we previously reported. Sham-operated (Sham) rats received similar surgery except the clip was not used. Systolic blood pressure (SBP) of tail artery was measured at weekly intervals in a conscious state with a noninvasive, computerized tail-cuff system. The criterion of hypertension is set as SBP ≥160 mmHg. Systolic blood pressure (SBP) of tail artery was simultaneously recorded with a PowerLab data acquisition system. The mRNA levels of IMD, CRLR, and RAMP1, 2, and 3 in the PVN were measured with real-time polymerase chain reaction and evaluated with SYBR Green I fluorescence. The protein levels of IMD, CRLR, and RAMP1, 2, and 3 in the PVN were measured with Western blotting. The effects of bilateral PVN microinjection of IMD (1, 5, or 25 pmol), CGRP receptor antagonist CGRP8-37 (0.2 nmol), AM receptor antagonist AM22-52 (1 nmol), nonselective NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME, 200 nmol), selective neuronal NOS (nNOS) inhibitor Nω-propyl-L-arginine hydrochloride (N-Propyl, 0.5 nmol), and endothelial NOS (eNOS) inhibitor N5-(1-iminoethyl)-l-ornithine dihydrochloride (L-NIO, 5 nmol) on RSNA and MAP in 2K1C rats were investigated. The effects of pretreatment with CGRP8-37, AM22-52, L-NAME, N-Propyl, or L-NIO on the RSNA and MAP responses to IMD were determined. NO metabolites (nitrate and nitrite, NOx) in the PVN were measured with a commercial assay kit (Cayman Chemical Co., Ann Arbor, MI). The effects of IMD and AM22-52 in the PVN on the NOx level were determined. Furthermore, the effects of chronic unilateral PVN infusion of IMD (0.5 nmol/µL, 1.32 nmol/d) or AM22-52 (20 nmol/µL, 52.8 nmol/d) on the SBP and HR with Alzet micro-osmotic pump (model 1004, Durect Corp, Cupertino, CA) were determined in conscious state. The pumps were implanted at the end of the fourth week after sham or 2K1C surgery, and the perfusion lasted for 15 days. SBP and HR were measured every 3 days in conscious state.

Because intracerebroventricular injection or NTS microinjection of IMD increased blood pressure and PNV microinjection of IMD decreased blood pressure, the integration of these varying effects was investigated. First, the effects of NTS pretreatment with AM22-52 on the RSNA and MAP responses to intracerebroventricular injection of IMD, and the effects of NTS microinjection of IMD on the RSNA and MAP responses to the PVN microinjection of IMD were determined in 2K1C rats. Second, the effects of PVN lesion induced by kainic acid (KA, 2 nmol) on the RSNA and MAP responses to the NTS microinjection of IMD, and the effects of NTS lesion on the RSNA and MAP responses to the PVN microinjection of IMD were examined in normal rats. Last, the effects of rostral ventrolateral medulla (RVLM) lesion on the RSNA and MAP responses to the NTS or PVN microinjection of IMD were investigated in normal rats.

IMD, CGRP8-37, and AM22-52 were obtained from Bachem (Hauptstrasse, Bubendorf, Switzerland). L-NAME and KA were purchased from Sigma Chemical Co (St. Louis, MO). N-Propyl and L-NIO were obtained from Tocris Bioscience (Bristol, United Kingdom). The chemicals used for PVN microinjection were dissolved in normal saline. IMD or AM22-52 used for PVN perfusion with micro-osmotic pump was dissolved in artificial cerebrospinal fluid.

Comparisons between 2 groups were made by Student t test. ANOVA followed by post hoc Bonferroni test was used when multiple comparisons were made. All data are expressed as means±SE. A value of P<0.05 was considered statistically significant. An expanded Methods section is available in the Methods in the online-only Data Supplement.

Results

General Data

Left ventricular weight, ratio of left ventricular weight to body weight, SBP, and MAP in 2K1C rats were significantly increased compared with Sham rats. There was no significant difference in body weight or HR between Sham and 2K1C rats at the end of the fourth week (Table).

IMD mRNA and Protein Expressions

IMD mRNA and protein expressions in the PVN were much lower in 2K1C rats than in Sham rats (Figure 1).

Effects of Different Doses of IMD

Microinjection of IMD into the PVN caused greater decreases in the RSNA and MAP in 2K1C rats than in Sham rats in a dose-related manner (Figure 2). The effects of IMD peaked at ≈30 minutes and lasted ≥60 minutes (Figure S1 in the online-only Data Supplement). Microinjection of IMD into the anterior hypothalamic area, which is adjacent to the PVN, had no significant effects on the RSNA and MAP in 2K1C rats.

Effects of CGRP8-37 and AM22-52

Microinjection of CGRP receptor antagonist CGRP8-37 into the PVN had no significant effects on RSNA and MAP in

Table. Body Weight, SBP, Baseline MAP, and Baseline HR at the End of the Fourth Week

<table>
<thead>
<tr>
<th>Variables</th>
<th>Sham</th>
<th>2K1C</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>114</td>
<td>126</td>
</tr>
<tr>
<td>BW, g</td>
<td>337±3</td>
<td>330±2</td>
</tr>
<tr>
<td>LVW, mg</td>
<td>538±3</td>
<td>766±9*</td>
</tr>
<tr>
<td>LWW/BW, mg/g</td>
<td>1.61±0.01</td>
<td>2.33±0.03*</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>119±1</td>
<td>187±2*</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>93±1</td>
<td>143±2*</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>356±4</td>
<td>367±4</td>
</tr>
</tbody>
</table>

Systolic blood pressure (SBP) was measured in conscious state by use of a noninvasive computerized tail-cuff system. Mean arterial pressure (MAP) and heart rate (HR) were measured under anesthesia with a pressure transducer through a catheter placed in the right carotid artery. Values are means±SE. 2K1C indicates 2-kidney 1-clip; BW, body weight; LVW, left ventricular weight; and Sham, sham operated.

*P<0.05 compared with the Sham.
both Sham and 2K1C rats. However, AM receptor antagonist AM22-52 significantly increased RSNA and MAP in 2K1C rats, but not in Sham rats (Figure 3). The sympathoinhibitory and depressor effects of IMD were almost abolished by pretreatment with AM22-52 but not with CGRP8-37 in the PVN in both Sham and 2K1C rats (Figure 4).

CRLR, RAMP1, RAMP2, and RAMP3 mRNA Expressions
CRLR and RAMP3 mRNA expressions in the PVN were higher in 2K1C rats than in Sham rats. RAMP1 mRNA expression was low in both Sham and 2K1C rats. There was no significant difference in RAMP1 and RAMP2 mRNA levels in the PVN between Sham and 2K1C rats (Figure 5).

CRLR, RAMP1, RAMP2, and RAMP3 Protein Expressions
CRLR, RAMP2, and RAMP3 protein expressions in the PVN were higher in 2K1C rats than in Sham rats. RAMP1 protein expression was low in both Sham and 2K1C rats. There was no significant difference in RAMP1 protein expression in the PVN between Sham and 2K1C rats (Figure 6).

Effects of NOS Inhibitor
Microinjection of nonselective NOS inhibitor L-NAME, selective nNOS inhibitor N-Propyl, or selective eNOS inhibitor L-NIO into the PVN significantly increased RSNA and MAP in both Sham and 2K1C rats (Figure 3). The sympathoinhibitory and depressor effects of IMD in the PVN were almost abolished by pretreatment with L-NAME and were attenuated by pretreatment with N-Propyl or L-NIO in Sham and 2K1C rats (Figure 4).

NOx Level in PVN
Microinjection of IMD into the PVN significantly increased NOx level in the PVN in 2K1C rats, but not in Sham rats. The effect of IMD on NOx level in the PVN was prevented by pretreatment with AM22-52 in 2K1C rats (Figure 7).

Effects of Chronic PVN Infusion of IMD and AM22-52 in Conscious State
Chronic unilateral PVN infusion of IMD significantly reduced both SBP and HR, whereas AM22-52 significantly increased SBP but not HR in conscious 2K1C rats. Either IMD or AM22-52 had no significant effects on the SBP and HR in conscious Sham rats (Figure 8).

Effects of IMD in Lateral Ventricles, NTS, and PVN
Injection of IMD into the lateral ventricles increased RSNA and MAP, which were attenuated by bilateral NTS
pretreatment with AM receptor antagonist AM22-52 in 2K1C rats (Figure S2). On the other hand, the inhibitory effects of IMD in the PVN on RSNA and MAP were attenuated by NTS pretreatment with IMD in 2K1C rats (Figure S3).

Effects of PVN Lesion and NTS Lesion
PVN lesion attenuated the sympathoexcitatory and pressor effects of IMD in NTS (Figure S4). However, NTS lesion had no significant effects on the sympathoinhibitory and depressor responses to IMD in the PVN (Figure S5).

Effects of RVLM Lesion
RVLM lesion almost abolished the sympathoexcitatory and pressor effects of IMD in the NTS (Figure S6) and the sympathoinhibitory and depressor effects of IMD in the PVN (Figure S7).

Discussion
Excessive sympathetic activity contributes to the pathogenesis of hypertension and the progression of organ damage, and intervention of the sympathetic activation has been considered as an important strategy for attenuating hypertension and related cardiac structural alterations. In the present study, either IMD mRNA or IMD protein was downregulated in the PVN in 2K1C rats. Microinjection of IMD into the PVN caused greater decreases in RSNA and MAP in 2K1C rats than in Sham rats. AM receptor antagonist AM22-52 in the PVN increased RSNA and MAP in 2K1C rats but not in Sham rats. It almost abolished the effects of IMD on the RSNA and MAP 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 sympathetic activity and reduces blood pressure via AM receptors rather than via CGRP receptors. Activation of the AM receptors by endogenous ligands in the PVN is not involved in the regulation of RSNA and MAP in physiological state but plays a tonic role in attenuating sympathetic outflow and blood pressure in renovascular hypertensive rats. The downregulation of IMD in the PVN contributes to the sympathoexcitatory activation and hypertension in 2K1C rats.
IMD, CGRP, and AM share the receptor system consisting of CRLR and RAMPs. CGRP binds to CRLR/RAMP1 complex, AM binds to CRLR/RAMP2 or CRLR/RAMP3 complex, but IMD binds to the 3 complexes nonselectively. In the present study, all the receptor components were expressed in the PVN. CRLR, RAMP2, and RAMP3 protein expressions and CRLR and RAMP3 mRNA expressions in the PVN were significantly increased in 2K1C rats compared with Sham rats. However, the RAMP1 mRNA and protein expressions were low in both Sham and 2K1C rats. The results suggest that the AM receptors (CRLR/RAMP2 or CRLR/RAMP3) rather than CGRP receptors (CRLR/RAMP1) were involved in the sympathetic activation and hypertension in 2K1C rats. The higher AM receptor expression and lower IMD expression in the PVN may be important in the pathogenesis of sympathetic activation in hypertension. Recently, various strategies such as noninvasive methods, including drug manipulation encompassing transformation into lipophilic analogs, prodrugs, chemical drug delivery, carrier-mediated drug delivery, and receptor-/vector-mediated drug delivery are widely used. The invasive methods with osmotic or biochemical means or direct drug delivery into the ventricles or brain regions are recognized. These advances provide a possibility for selective intervention of IMD expression or CRLR/RAMPs system in the PVN. It is speculated that the increased IMD expression in the PVN may be beneficial for the attenuation of hypertension. It is well documented that renin–angiotensin system contributes to hypertension, and angiotensin receptor blockers are commonly used as antihypertensive agents. Angiotensin II in the PVN caused greater increases in RSNA and MAP in 2K1C rats than in Sham rats, whereas angiotensin II type 1 (AT) receptor antagonist losartan reduced RSNA and MAP in 2K1C rats. Furthermore, angiotensin II and AT, receptor expressions in the PVN are increased in 2K1C rats. IMD is known to inhibit rat cardiac fibroblast activation induced by angiotensin II. However, it is not known whether the downregulation of IMD in the PVN is caused by elevated blood pressure or increased angiotensin II. The upregulation of CRLR,

RAMP2, and RAMP3 may be an important compensatory change attributable to a lower IMD expression in the PVN in hypertension. The upregulated AM receptors contribute to the long-lasting and profound sympathetic inhibition and depressor effects of IMD in the PVN of 2K1C rats.

It is known that NO in the PVN inhibits the RSNA response to angiotensin II and the firing activity of PVN neurons that innervate the medulla oblongata. Gene transfer of neuronal NOS to the PVN decreased the enhanced RSNA and MAP responses to NOS inhibitor in rats with chronic heart failure. It is known that the peripheral vasodilator effects of IMD are mediated by AM receptors and NO but not by ATP-sensitive potassium channels. AM-induced depressor effect in the PVN is mediated by NO, and nicotinamide adenine dinucleotide phosphate diaphorase (a marker of nNOS-containing neurons) and RAMP2 (an AM receptor component) double-labeled neurons were found in both parvocellular and magnocellular subdivisions of the PVN. We found that microinjection of IMD into the PVN increased NOx level in 2K1C rats, which was abolished by the pretreatment with AM22-52. Nonselective NOS inhibitor in the PVN prevented and selective nNOS inhibitor or eNOS inhibitor attenuated the IMD-induced decreases in the RSNA and MAP in both Sham and 2K1C rats. The results indicate that NO in the PVN mediates the effects of IMD on the RSNA and MAP. Both nNOS and eNOS in the PVN are involved in the effects of IMD, which was supported by the finding that the AM-induced
Depressor effect in the PVN is mediated by NO from nNOS and eNOS. It seemed that the effects of IMD were not completely abolished by the AM receptor antagonist or NOS inhibitors, suggesting a possibility that some other downstream molecules such as undetermined type of receptors are involved in mediating the effects of IMD. Previous studies showed that PVN microinjection of α-CGRP increased blood pressure and sympathetic outflow. However, AM caused depressor effect, which was similar to the effect of IMD in the present study. The results suggest that the effects of AM22-52 on the baseline RSNA and MAP may be related to the blockade of both IMD and AM. Chronic PVN infusion of IMD reduced and that of AM22-52 increased blood pressure in conscious 2K1C rats. The effect was greater in 2K1C rats than in Sham rats in conscious state, being similar to the effect in anesthetized rats. One possibility to consider is that IMD and AM receptors in the PVN play a tonic role in regulating blood pressure in normotensive rats, and the compensatory increase in AM receptors in the PVN may contribute to the greater depressor effect of IMD and the greater pressor effect of AM22-52 in 2K1C rats than in Sham rats.

Presynaptic efferent projections from the PVN target RVLM, NTS, and sympathetic preganglionic neurons in the spinal cord. PVN presynaptic neurons receive afferent projections from the NTS, which innervate GABAergic neurons in the caudal ventrolateral medulla, which, in turn, innervate neurons in the RVLM. It was reported that intracerebroventricular injection of IMD caused a pressor effect and sympathetic activation. We recently found that microinjection of IMD into the NTS, which is near to the fourth ventricle, increased RSNA and MAP. In the present study, blockade of AM receptors in the NTS attenuated the effects of intracerebroventricular injection of IMD, suggesting that NTS may be one of main action sites of IMD injected into the lateral ventricles. KA is commonly used as a tool to destroy neuronal perikarya selectively without damage of axons of passage and terminals. No neuronal firing can be observed at the KA injection site 1 hour after KA administration. Our previous study showed that bilateral PVN microinjection of KA abolished cardiac sympathetic afferent reflex and adi- pose afferent reflex in rats. The effectiveness of KA-induced lesion was confirmed by the prominent vacuolation and dilatation of the mitochondria in the neuronal perikarya at the

Figure 7. Nitric oxide metabolite (NOx) level in the paraventricular nucleus (PVN). A, Effects of PVN microinjection of saline or intermedin (IMD, 25 pmol) on the NOx level in the PVN in sham-operated (Sham) and 2-kidney 1-clip (2K1C) rats; B, Effects of IMD-pretreated with saline or adrenomedullin receptor antagonist AM22-52 (1 nmol) on the NOx level in the PVN in 2K1C rats. IMD was administered 10 minutes after the pretreatment. Values are mean±SE. *P<0.05 vs Sham; †P<0.05 vs IMD; ‡P<0.05 vs saline+IMD. n=6 for each group.

Figure 8. Effects of chronic unilateral paraventricular nucleus (PVN) infusion of intermedin (IMD, 0.5 nmol/µL, 1.32 nmol/d) or adrenomedullin receptor antagonist AM22-52 (20 nmol/µL, 52.8 nmol/d) for 15 consecutive days on the systolic blood pressure (SBP; A) and heart rate (HR; B) in conscious state. Values are mean±SE. *P<0.05 vs Sham; †P<0.05 vs artificial cerebrospinal fluid (ACSF). n=6 for each group. 2K1C indicates 2-kidney 1-clip; and Sham, sham operated.
injection sites 100 minutes after KA administration and the considerable reduction in the number of neurons at the injection sites 2 days after KA administration. PVN lesion with KA only attenuated the effects of IMD in the NTS, and NTS lesion did not affect the effects of IMD in the PVN. RVLVM lesion almost abolished the effects of IMD in the NTS or in the PVN. Furthermore, microinjection of IMD into the NTS neutralized the effects of PVN microinjection of IMD. These results suggest that both PVN and RVLVM contribute to the integration of the sympathoinhibitory and depressor effects of IMD in the PVN and the sympathoexcitatory and pressor effects in the NTS.

Perspectives

It is known that excessive sympathetic activity contributes to hypertension and related organ damage in 2K1C rats. The present study found that IMD in the PVN attenuated sympathetic outflow and decreased blood pressure in 2K1C-induced renovascular hypertension, which were mediated by the AM receptors in the PVN. Blockade of AM receptors in the PVN increased sympathetic outflow and blood pressure in 2K1C rats but not in Sham rats. IMD in the PVN was downregulated, whereas its receptor components CRLR, RAMP2, and RAMP3 were upregulated in 2K1C rats. IMD increased NOx production and NOX receptors in the PVN. Blockade of AM receptors in the PVN abolished the effects of IMD on the RSNA and MAP. These results indicate that IMD in the PVN plays sympathoinhibitory and antihypertensive roles in 2K1C rats, which are mediated by AM receptors (CRLR/RAMP2 or CRLR/RAMP3) and their downstream NO.

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Disclosures

None.

References


**Novelty and Significance**

**What Is New?**
- Intermedin (IMD) in the paraventricular nucleus (PVN) decreased sympathetic outflow and blood pressure in 2-kidney 1-clip rats, which were mediated by adrenomedullin receptors in the PVN.
- IMD in the PVN was downregulated, whereas its receptor components calcitonin receptor–like receptor and receptor activity–modifying proteins 2 and 3 were upregulated in 2-kidney 1-clip rats.
- IMD increased nitric oxide metabolites level in the PVN, and nitric oxide mediated the effects of IMD in the PVN on the renal sympathetic nerve activity and mean arterial pressure in 2-kidney 1-clip rats.

**What Is Relevant?**
- Downregulated IMD in the PVN contributes to the sympathetic activation in hypertension.
- Activation of adrenomedullin receptors in the PVN attenuates sympathetic activity and blood pressure in hypertension.

**Summary**

IMD in the PVN inhibits sympathetic activity and attenuates hypertension in 2-kidney 1-clip rats, which are mediated by adrenomedullin receptors (calcitonin receptor–like receptor/receptor activity–modifying protein 2 or calcitonin receptor–like receptor/receptor activity–modifying protein 3) and their downstream nitric oxide.
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Intermedin in paraventricular nucleus attenuates sympathetic activity and blood pressure via nitric oxide in hypertensive rats

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Supplemental Methods

Renovascular hypertension model

Renovascular hypertension was induced in rat weighing 160-180 g with two-kidney one-clip (2K1C) method as we previously reported.1,2 Briefly, the rat was anaesthetized by pentobarbital sodium (60 mg/kg) intraperitoneally. The adequacy of anaesthesia was evaluated by the loss of a pedal withdrawal reflex. A right retroperitoneal flank incision was performed with sterile techniques. Right renal artery was exposed, and partly occluded with an U-shaped silver clip with an internal diameter of 0.20 mm. Sham-operated rats (Sham) received similar surgical process except using the clip. The criterion of hypertension is set as systolic arterial pressure (SBP) of tail artery was greater than or equal to 160 mmHg in a conscious state. Four rats were excluded because their SBP was not high enough to meet the criterion.

SBP measurement

SBP of tail artery was measured weekly in a conscious state with a non-invasive computerized tail-cuff system (NIBP, ADInstruments, Sydney, Australia). The rats were warmed for 10-20 min at 28°C before the measurements to allow detection of tail artery pulsations and to achieve a steady pulse level. SBP was obtained by averaging 10 measurements at weekly intervals. To minimize the stress-induced SBP fluctuation, the rat was trained by measuring SBP daily for at least 10 days before 2K1C or sham operation.3,4

General procedures of acute experiment

Acute experiment was carried out at the end of the 4th week after surgery. Rats were anesthetized with urethane (800 mg/kg) and α-chloralose (40 mg/kg) intraperitoneally. Supplemental doses of anesthesia were used during the experiment to maintain an appropriate level of anaesthesia that was assessed by the absence of corneal reflexes and paw withdrawal response to a noxious pinch. A midline incision in the neck was made to expose the trachea and carotid artery. The trachea was intubated and connected to a rodent ventilator (Model 683, Harvard Apparatus Inc, Holliston, MA, USA) for mechanical ventilation. Mean arterial pressure (MAP) and heart rate (HR) were measured with a pressure transducer via a catheter in the right carotid artery. The MAP, HR and renal sympathetic nerve activity (RSNA) were simultaneously recorded with a PowerLab data acquisition system (8/35, ADInstruments, Castle Hill, Australia).

PVN microinjection

Stereotaxic coordinates for PVN were 1.8 mm caudal from bregma, 0.4 mm lateral to midline and 7.9 mm ventral to dorsal surface.3 Microinjection volume was 50 nl for each side of PVN and the microinjections were completed within 1 min. At the end of the experiment, same volume of Evans Blue (2%) was injected into the microinjection site for histological identification. Rats with microinjection sites outside the PVN were excluded from data analysis.

Intracerebroventricular (icv) injection

The coordinates for lateral ventricles were 0.8 mm caudal from bregma, 1.4 mm lateral
to midline and 3.8 mm ventral to dorsal surface. Microinjection volume was 500 nl for each side of lateral ventricles. The microinjections were completed within 1 min. At the end of the experiment, same volume of Evans Blue (2%) was injected into the microinjection site for histological identification. Rats with microinjection sites outside the lateral ventricles were excluded from data analysis.

**NTS microinjection**

Rat skull was bent downward to a 45° angle from the horizontal plane of the stereotaxic instrument, and the dorsal surface of the medulla was exposed by a limited occipital craniotomy. Calamus scriptorius was used as a landmark for guiding the injection site. The coordinates for nucleus tractus solitarii (NTS) were 0.2 mm rostral to 0.3 mm caudal, 0–0.4 mm lateral, and 0.3–0.5 mm deep. Microinjection volume was 50 nl for each side of NTS and the microinjections were completed within 1 min. The NTS was functionally identified by a depressor response of at least 25 mm Hg to injection of 2 nmol of L-glutamate. At the end of the experiments, same volume of Evans Blue (2%) was injected into the microinjection site for histological identification. Rats with microinjection sites outside the NTS were excluded from data analysis.

**RVLM microinjection**

The coordinates for the rostral ventrolateral medulla (RVLM) are 4.5-5.0 mm posterior to lambda, 1.8-2.1 mm lateral to midline, and 8.1-8.4 mm below the dorsal surface of cerebellum. Microinjection volume was 50 nl for each side of RVLM and the microinjections were completed within 1 min. The RVLM was functionally identified by a pressor response of at least 25 mm Hg to injection of 2 nmol of L-glutamate. The histological identification was made to verify each microinjection site. At the end of the experiments, same volume of Evans Blue (2%) was injected into the microinjection site for histological identification. Rats with microinjection sites outside the RVLM were excluded from data analysis.

**RSNA recording**

Left renal sympathetic nerve was isolated through a retroperitoneal incision, and was cut distally to eliminate its afferent activity. The nerve was immersed in warm mineral oil, and placed on a pair of silver electrodes. The RSNA was amplified with a four channel AC/DC differential amplifier (DP-304, Warner Instruments, Hamden, CT, USA) with a high pass filter at 10 Hz and a low pass filter at 3,000 Hz. 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.

**PVN sample preparation**

The rat was anaesthetized with an overdose of pentobarbital (100 mg/kg, i.p.). The brain of the rat was quickly removed, frozen in liquid nitrogen and stored at -80 °C until being sectioned. Brain tissue was cut into 450 µm coronal section within the levels from 1.5 mm to 2.0 mm caudal from bregma. A punch biopsy was obtained from bilateral PVN using a 15-gauge needle (inner diameter 1.5 mm) with frozen microtome (Leica CM1900-1-1, Wetzlar, Hessen, Germany).
Real-time PCR for measurement of IMD, CRLR, RAMP1, 2 and 3 mRNA in PVN

The mRNA levels of intermedin (IMD) and its receptor components calcitonin receptor-like receptor (CRLR), receptor activity-modifying proteins (RAMP) 1, 2 and 3 in the PVN were measured. Simply, total RNA of PVN was extracted by standard techniques and reversely transcribed using a reverse-transcription system (Promega, Madison, WI, USA). The rat primers used for real-time PCR were as follows.

IMD primer: forward 5'-CCCTACCTCGCCCTGTAGTCT-3', reverse 5'-ACCCACCTCAGCCATAACTGT-3';
CRLR primer: forward 5'-CAACTGCTGGATCAGCTCAG-3', reverse 5'-CATCGCTGATTGTTGACACC-3';
RAMP1 primer: forward 5'-CAATCCGGAAGTGGACAAGT-3', reverse 5'-GACACCTACACGATGCCCTCT-3';
RAMP2 primer: forward 5'-CAAGGACTGGTGCAACTGGAC-3', reverse 5'-ACACCACAAGCGTAACGAGGA-3';
RAMP3 primer: forward 5'-ACCTGTCGGAGTTCATCGTGT-3', reverse 5'-TAGCCACGGTGCAACAAGACTG-3';
β-actin primer: forward 5'-GAGACCTTCAACACCCAGCC-3', reverse 5'-TCGGGGCATCGGAACCGCTCA-3'.

After being denatured at 95 °C for 7 min, the solution underwent PCR for IMD, CRLR, RAMP1, RAMP2, RAMP3 and β-actin at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 45 s for 40 cycles. The measurements of mRNA of IMD, CRLR, RAMP1, 2 and 3 were carried out with Multiplex Quantitative PCR System (M93000, ratagene, La Jolla, CA, USA) and evaluated with SYBR Green I fluorescence.9

Western blotting for measurement of IMD, CRLR, RAMP1, 2 and 3 protein in PVN

Total protein of PVN in the homogenate supernatant was extracted and measured using a protein assay kit. The CRLR, RAMP1, RAMP2, RAMP3 and β-actin protein expression in the PVN was determined with Western blotting method. Briefly, after processes of electrophoresis and transmembrane, the proteins on nitrocellulose membrane were probed with the use of the primary antibodies (Santa Cruz Biotechnology, Inc., U.S.A.) of IMD (1:1000), CRLR (1:2000), RAMP1 (1:1000), RAMP2 (1:1000), RAMP3 (1:1000) and GAPDH (1:4000). This was followed by incubation with horseradish peroxidase–conjugated goat anti-rabbit IgG. GAPDH (Bioworld Technology Inc., USA) was used as a loading control.9

Measurement of NO metabolites (NOx) level in PVN

Nitric oxide metabolites (NOx) level is widely used as an index of NO level.10-12 NO production in the PVN was evaluated by the determination of the concentration of its stable metabolites nitrates/nitrites by using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Co., Ann Arbor, Michigan, USA). Total protein of PVN in the homogenate supernatant was extracted and the supernatant was ultrafiltered, then the eluant was used to measure NOx level according to manufacturer’s instructions. The absorbance of the samples was measured by a microreader in the wavelength of 540 nm. The nitrite concentration of samples was determined by comparison with the
nitrite standard reference curve. Results are expressed as nmol/mg of protein.

**PVN infusion with micro-osmotic pump**

Alzet micro-osmotic pump and brain infusion kit (model 1004, Durect Corp, Cupertino, CA, USA) were used for unilateral PVN infusion at the end of the 4th week after sham or 2K1C surgery. The rat was anaesthetized with pentobarbital (50 mg/kg, i.p.) for the surgery of pump implantation. The pump was connected to a cannula using 5 cm of vinyl tubing. The cannula was stereotactically placed into the PVN with the same coordinates to the PVN microinjection described above. The pump was implanted subcutaneously over the scapula. The perfusion lasted for 15 days. SBP and HR were measured every 3 days in conscious state. The chemicals used for the perfusion were dissolved in artificial cerebrospinal fluid (ACSF), and the vehicle (ACSF) was used as a control.

**Experimental design**

**Experiment 1:** IMD, CRLR, RAMP1, RAMP2 and RAMP3 mRNA in the PVN was determined with real-time PCR in both Sham and 2K1C rats (n= 4 for each group). Because the PVN tissues are small, the measurement for mRNA levels and corresponding protein levels in the PVN can not be carried out in the same animals.

**Experiment 2:** IMD, CRLR, RAMP1, RAMP2 and RAMP3 protein expression in the PVN was determined with Western blotting method in both Sham and 2K1C rats (n= 4 for each group).

**Experiment 3:** Effects of IMD, CGRP receptor antagonist CGRP8-37, AM receptor antagonist AM22-52, non-selective nitric oxide synthase (NOS) inhibitor NG-nitro-L-arginine methyl ester (L-NAME), selective nNOS inhibitor Nω-propyl-L-arginine hydrochloride (N-Propyl) and eNOS inhibitor N(5)-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO) on the RSNA and MAP were investigated. The PVN microinjection of saline, three doses of IMD (1, 5 or 25 pmol), CGRP8-37 (0.2 nmol), AM22-52 (1 nmol), L-NAME (200 nmol), N-Propyl (0.5 nmol) or L-NIO (5 nmol) was carried out in 9 groups of Sham rats and 9 groups of 2K1C rats (n=6 for each group). To exclude the possibility that the effects of IMD were caused by diffusion to other brain area, the effects of microinjection of IMD (25 pmol) into the anterior hypothalamic area which is adjacent to the PVN were determined in 2K1C rats (n= 3). The doses of chemicals were selected according to our preliminary study and previous studies.14-16

**Experiment 4:** Effects of pretreatment with the PVN microinjection of saline, CGRP8-37 (0.2 nmol), AM22-52 (1 nmol), L-NAME (200 nmol), N-Propyl (0.5 nmol) or L-NIO (5 nmol) on the RSNA and MAP responses to the PVN microinjection of IMD (25 pmol) were investigated in 6 groups of Sham rats and 6 groups of 2K1C rats (n=6 for each group). The IMD was administered 10 min after the pretreatment.

**Experiment 5:** Effects of the PVN microinjection of saline or IMD (25 pmol) on the NOx level in the PVN were investigated in Sham and 2K1C rats (n=6 for each group). The NOx level in the PVN was measured 30 min after the microinjection.

**Experiment 6:** Effects of pretreatment with the PVN microinjection of saline or AM22-52 (1 nmol) on the NOx response to the PVN microinjection of IMD into the PVN in 2K1C rats (n=6 for each group). IMD was administered 10 min after the
pretreatment. NOx level in the PVN was measured 30 min after the microinjection of IMD.

Experiment 7: Effects of unilateral PVN infusion of ASCF, IMD (0.5 nmol/µl, 1.32 nmol/day) or AM22-52 (20 nmol/µl, 52.8 nmol/day) with micro-osmotic pump on the SBP and HR were determined in 3 groups of Sham rats and 3 groups of 2K1C rats (n=6 for each group). The perfusion lasted for 15 days. SBP and HR were measured every 3 days in conscious state.

Experiment 8: Previous studies showed that intracerebroventricular (icv) injection or microinjection of IMD into the NTS increased blood pressure and sympathetic outflow, while present study showed microinjection of IMD into the PVN decreased blood pressure and sympathetic outflow. The experiment was designed to determine whether NTS mediated the effects of icv injection of IMD. The RSNA and MAP responses to the icv injection of IMD (250 pmol) were determined 10 min after the NTS pretreatment with saline or AM receptor antagonist AM22-52 (1 nmol) in 2K1C rats (n=5 for each group).

Experiment 9: The experiment was to investigate whether IMD in the NTS neutralized the effects of IMD in the PVN. The effects of NTS microinjection of saline or IMD (25 pmol) on the RSNA and MAP responses to the PVN injection of IMD (25 pmol) were determined in 2K1C rats (n=4 for each group).

Experiment 10: Kainic acid (KA) is commonly used as a tool to destroy neuronal perikarya selectively without damage of axons of passage and terminals. No neuronal firing can be observed at the KA injection site one hour after KA administration. Our previous study showed that bilateral PVN microinjection of KA abolished cardiac sympathetic afferent reflex (CSAR) and adipose afferent reflex (AAR) in rats. The effectiveness KA-induced lesion was confirmed by the prominent vacuolation and dilatation of the mitochondria in the neuronal perikarya at the injection sites 100 min after KA administration, and the considerable reduction in the number of neurons at the injection sites 2 days after KA administration. This experiment was designed to determined the interaction of PVN and NTS. The effects of PVN pretreatment with saline or KA (2 nmol) on the RSNA and MAP responses to the NTS microinjection of IMD (25 pmol), and the effects of NTS pretreatment with saline or KA (2 nmol) on the RSNA and MAP responses to the PVN microinjection of IMD (25 pmol) were determined in normal rats (n=5 for each group), respectively. The microinjections of IMD were carried out 120 min after KA pretreatment.

Experiment 11: The effects of RVLM pretreatment with saline or KA (2 nmol) on the RSNA and MAP responses to the NTS or PVN microinjection of IMD (25 pmol) were determined in normal rats (n=5 for each group), respectively. The microinjections of IMD were carried out 120 min after KA pretreatment.

References


Supplemental Figure

Figure S1. Representative tracings showing the effects of PVN microinjection of saline or intermedin (IMD, 25 pmol) on the arterial blood pressure (ABP), mean arterial pressure (MAP) and renal sympathetic nerve activity (RSNA) in 2K1C-induced renovascular rats.

Figure S2. Effects of NTS microinjection of saline or AM receptor antagonist AM22-52 (1 nmol) on the RSNA and MAP as well as RSNA and MAP responses to icv injection of IMD (250 pmol) in 2K1C rats. Values are mean±SE. * P<0.05 vs. Saline(NTS); † P<0.05 vs. Saline(NTS)+IMD(icv). n=5 for each group.
Figure S3. Effects of NTS microinjection of saline or IMD (25 pmol) on the RSNA and MAP as well as RSNA and MAP responses to the PVN microinjection of IMD (25 pmol) in 2K1C rats. Values are mean±SE. * P<0.05 vs. Saline(NTS); † P<0.05 vs. Saline(NTS)+IMD(PVN). n=4 for each group.

Figure S4. Effects of PVN microinjection of saline or kainic acid (2 nmol) on the RSNA and MAP responses to the NTS microinjection of IMD (25 pmol) in normal rats. The microinjections of IMD were carried out 120 min after KA pretreatment. Values are mean±SE. * P<0.05 vs. Saline(PVN)+IMD(NTS). n=5 for each group.
Figure S5. Effects of NTS microinjection of saline or kainic acid (2 nmol) on the RSNA and MAP responses to the PVN microinjection of IMD (25 pmol) in normal rats. The microinjections of IMD were carried out 120 min after KA pretreatment. Values are mean±SE. * P<0.05 vs. Saline(NTS)+IMD(PVN). n=5 for each group.

Figure S6. Effects of RVLM microinjection of saline or kainic acid (2 nmol) on the RSNA and MAP responses to the NTS microinjection of IMD (25 pmol) in normal rats. The microinjections of IMD were carried out 120 min after KA pretreatment. Values are mean±SE. * P<0.05 vs. Saline(RVLM)+IMD(NTS). n=5 for each group.
Figure S7. Effects of RVLM microinjection of saline or kainic acid (2 nmol) on the RSNA and MAP responses to the PVN microinjection of IMD (25 pmol) in normal rats. The microinjections of IMD were carried out 120 min after KA pretreatment. Values are mean±SE. * P<0.05 vs. Saline(RVLM)+IMD(PVN). n=5 for each group.