Neurosci. Bull. February, 2019, 35(1):34–46
https://doi.org/10.1007/s12264-018-0292-9

www.neurosci.cn
www.springer.com/12264

ORIGINAL ARTICLE

Intermedin in Paraventricular Nucleus Attenuates Sympathoexcitation and Decreases TLR4-Mediated Sympathetic Activation *via* Adrenomedullin Receptors in Rats with Obesity-Related Hypertension

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Received: 9 April 2018 / Accepted: 14 June 2018 / Published online: 1 October 2018

Abstract Intermedin/adrenomedullin-2 (IMD/AM2), a member of the calcitonin gene-related peptide/AM family, plays an important role in protecting the cardiovascular system. However, its role in the enhanced sympathoexcitation in obesity-related hypertension is unknown. In this study, we investigated the effects of IMD in the paraventricular nucleus (PVN) of the hypothalamus on sympathetic nerve activity (SNA), and lipopolysaccharide (LPS)-induced sympathetic activation in obesity-related hypertensive (OH) rats induced by a high-fat diet for 12 weeks. Acute experiments were performed under anesthesia. The dynamic alterations of sympathetic outflow were evaluated as changes in renal SNA and mean arterial pressure (MAP) in response to specific drugs. Male rats were fed a control diet (12% kcal as fat) or a high-fat diet (42% kcal as fat) for 12 weeks to induce OH. The results showed that IMD protein in the PVN was downregulated, but Toll-like receptor 4 (TLR4) and plasma norepinephrine (NE, indicating sympathetic hyperactivity) levels, and systolic blood pressure were increased in OH rats. LPS (0.5 μg/50 nL)-induced enhancement of renal SNA and MAP was greater in OH rats than in obese or control rats. Bilateral PVN microinjection of IMD (50 pmol) caused greater decreases in renal SNA and MAP in OH rats than in control rats, and inhibited LPS-induced sympathetic activation, and these were effectively prevented in OH rats by pretreatment with the AM receptor antagonist AM22-52. The mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) inhibitor U0126 in the PVN partially reversed the LPS-induced enhancement of SNA. However, IMD in the PVN decreased the LPS-induced ERK activation, which was also effectively prevented by AM22-52. Chronic IMD administration resulted in significant reductions in the plasma NE level and blood pressure in OH rats. Moreover, IMD lowered the TLR4 protein expression and ERK activation in the PVN, and decreased the LPS-induced sympathetic overactivity. These results indicate that IMD in the PVN attenuates SNA and hypertension, and decreases the ERK activation implicated in the LPS-induced enhancement of SNA in OH rats, and this is mediated by AM receptors.

Keywords Intermedin · Sympathoexcitation · Obesity-related hypertension · Paraventricular nucleus · Toll-like receptor 4

Introduction

It is well known that obesity and hypertension independently increase the risk of cardiovascular morbidity and mortality. Obesity-related hypertension (OH) has emerged as one of the most common chronic diseases worldwide. Excess weight gain involves enhanced sympathetic nervous system activity, which has been shown to contribute to the development of obesity-related hypertension [1–3].

The hypothalamic paraventricular nucleus (PVN), the most important cardiovascular regulatory center in the brain, plays a major role in regulating sympathetic outflow for the control of blood pressure (BP) [4–6]. OH is a chronic inflammatory condition, and evidence suggests that

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increased inflammation in the PVN plays an important role in its development [7–10]. Toll-like receptors (TLRs) are critical components of the innate immune system [7–10], but their role in the etiology of OH is unclear. Out of 13 TLRs, TLR4 has been implicated in the pathogenesis of hypertension [11–13]. Lipopolysaccharide (LPS) can act on TLR4 to elicit an inflammatory response [14], and TLR4 blockade in the PVN attenuates inflammation, mean arterial pressure (MAP), and sympathetic nerve activity (SNA) in hypertensive rats [11]. Given the importance of the PVN in initiating high BP, it is necessary to investigate the effects of TLR4 in the PVN on SNA and BP in OH. In addition, the underlying molecular mechanisms in the TLR4-mediated sympathetic response in OH have never been studied.

Adrenomedullin (AM) 2/intermedin (IMD), a short peptide, belongs to the calcitonin gene-related peptide (CGRP) superfamily [15–17]. It shares receptors (the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes) with CGRP and AM, and has protective roles in cardiovascular diseases [15–17]. The PVN is also an important integrative site implicated in the neurogenic component of high-fat diet (HFD)-induced sympathetic activation and hypertension in OH rats [4, 7, 8, 18], and IMD, CGRP, and AM receptors have been found in the PVN [19–21]. It is well known that IMD has potent anti-inflammatory effects [22–24]. For instance, treatment with IMD inhibits chronic inflammation in adipose tissue, and improves systemic insulin sensitivity in mice with hyperhomocysteinemia [23]. However, its effect on sympathetic overactivity aggravated by inflammation remains largely unknown. So it is imperative to investigate the role of IMD on SNA and BP in animal models with OH. A recent study in our laboratory has shown that microinjection of IMD into the PVN decreases renal SNA, and chronic PVN infusion of IMD reduces BP in conscious 2-kidney 1-clip rats [25]. Therefore, it is of interest to determine the roles and mechanisms of IMD in the PVN in sympathetic activation and hypertension in OH.

The present study was designed to determine whether IMD in the PVN attenuates sympathetic activation and hypertension, and which receptors in the PVN are implicated in the effects of IMD in OH rats. Furthermore, we explored the role of IMD in the TLR4 activation by LPS within the PVN that leads to sympathoexcitation and increased BP.

Materials and Methods

Experimental Design

A control diet or HFD were administered to rats for 12 weeks. Body weight and systolic blood pressure (SBP) were assessed in the conscious state. Acute experiments were carried out at the end of week 12. PVN injection sites were identified histologically in all injected rats.

Experiment 1

The plasma NE levels (n = 6 for each) and IMD and TLR4 protein expression in the PVN (n = 3–4 for each) were determined in control and OH rats.

Experiment 2

The change of SNA was evaluated by the renal SNA and MAP responses to IMD, LPS, an ERK inhibitor, or an AM receptor antagonist in control and OH rats. Each rat received PVN microinjection. The intervals between applications of drugs were at least 4 h to allow complete recovery.

Experiment 3

The effects of pretreatments consisting of PVN microinjections of saline, IMD (50 pmol/L), AM22-52 (1 nmol/L), CGRP8-37 (0.2 nmol/L), and the MAPK/ERK inhibitor U0126 (50 μmol/L) on the baseline renal SNA and MAP responses to PVN microinjection of LPS (0.5 μg/50 nL) were investigated in OH rats. The PVN microinjection of LPS was carried out 10 min after pretreatment with saline, IMD, or U0126, and PVN microinjection of IMD was carried out 10 min after pretreatment with AM22-52 or CGRP8-37.

Experiment 4

We determined the effects of chronic systemic infusion of IMD [300 ng/kg per hour, with an Alzet micro-osmotic pump (model 2004, Durect Corp., Cupertino, CA)] on the SNA, BP, TLR4 protein expression and ERK activation in the PVN, and LPS-induced sympathoexcitation in OH rats. The pumps were implanted in OH rats at the end of week 12, and the perfusion lasted for 4 weeks as the HFD was continued.

Animals

Male Sprague-Dawley rats weighing 160–180 g were randomly divided into two groups: one received a control diet (12% kcal as fat: 12% fat, 60% carbohydrate, 28% protein), and the other received an HFD (42% kcal as fat: 42% fat, 43% carbohydrate, 15% protein) for 12 or 16 weeks. The diets were from Trophic Animal Feed High-tech Co. Ltd (Nantong, China). The experimental procedures were approved by the Animal Experimental Ethics Committee of Animal Core Facility of Nanjing Medical University and complied with the Guide for the Care and Use of Laboratory Animals (NIH publication, Eighth

edition, 2011). All rats were housed under a 12-h light-dark cycle, in a temperature- and humidity-controlled room. After 12 weeks, the rats consuming the HFD were ranked based on weight gain and SBP [4]. Obesity-related hypertensive rats (OH group) with higher weight gains and an SBP $\geq$ 140 mm Hg, and obese rats (OB group) with higher weight gains and an SBP $<$ 140 mm Hg were used. The rats receiving the control diet served as a control group.

Measurement of SBP

SBP was measured from the tail artery with a noninvasive computerized tail-cuff system (NIBP, ADInstruments, Sydney, Australia). The rats were warmed for $\sim$ 20 min at 28 °C to obtain a steady pulse level. Before performing acute experiments, the rats were trained by measuring SBP daily for at least 10 days to minimize fluctuations, and the value was the average of 10 measurements [25].

Blood Preparation

At the end of 12 or 16 weeks, $\sim$ 1.5 mL of blood from the tail tip of each conscious rat was collected for measuring the levels of NE, glucose, insulin, triglycerides, and cholesterol. The plasma was stored at $-$ 80 °C until use.

Measurement of Plasma Glucose, Insulin, Cholesterol, and Triglyceride Levels

At the end of 12 or 16 weeks, all rats were fasted overnight and blood was collected as above. Following the manufacturer's instructions, the glucose oxidase method was used to measure the fasting plasma glucose content with a kit (Jiancheng Bioengineering, Nanjing, China), the enzyme-linked immunoassay method was used to assess the fasting plasma insulin levels with a kit (USCN Business Co., Ltd., Wuhan, China), and a colorimetric assay was used for cholesterol and triglyceride measurements with kits (Jiancheng Bioengineering, Nanjing, China).

Measurement of Plasma NE Levels

A 96-well plate (USCN Business Co., Ltd) with specific antibodies was used for rat NE detection. First, a standard diluent buffer and the samples were added to the plates, incubated overnight, and washed 5 times. A horseradish peroxidase-conjugated solution was added and incubated for 1 h, then washed out 5 times. Finally, stop solution was added to stop the reactions, and the final solution was read at 450 nm on a microplate reader (ELX800, BioTek Instruments Inc., Winooski, VT).

Measurement of Protein Expression Levels

The protein expression levels of IMD, TLR4, p38, phosphorylated JNK and ERK, and total p38, JNK, and ERK in the PVN were measured as previously reported [25]. Briefly, total PVN proteins in homogenates were extracted and measured. Western blot analyses were carried out using rabbit polyclonal antibodies against IMD (USCN Business Co., Ltd.), TLR4, p38, phosphorylated JNK and ERK, total p38, JNK, and ERK (Proteintech Group, Inc. Rosemont, IL), and GAPDH (Bioworld Technology, Louis Park, MN) as the primary antibodies. The peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a secondary antibody. The levels of IMD and TLR4 were expressed as ratios to the GAPDH protein levels.

General Procedures of Acute Experiments

After 12 or 16 weeks, the rats were anesthetized with intraperitoneal urethane (800 mg/kg) and α-chloralose (40 mg/kg). Through a midline incision, the trachea and carotid artery were exposed, then the cannulated trachea was connected to a rodent ventilator (Model 683, Harvard Apparatus Inc., Holliston, MA), and the cannulated right carotid artery was used to record MAP. Using a PowerLab data acquisition system (8/35, ADInstruments), the renal SNA and MAP were recorded simultaneously. To maintain an adequate depth of anesthesia, supplemental doses of anesthetics were administered.

Vagotomy and Baroreceptor Denervation

In order to minimize the confounding activity of the baroreflex on SNA and BP, vagotomy and baroreceptor denervation were carried out as previously described [25].

Renal SNA Recording

Dynamic alterations in sympathetic outflow were assessed by renal SNA. Briefly, through a retroperitoneal incision, the left renal sympathetic nerve was isolated and cut distally to abolish afferent activity. Then, the nerve was placed on a pair of silver electrodes and immersed in mineral oil. An AC/DC differential amplifier (Model 3000; A-M Systems, Washington, DC) was used to amplify the renal SNA, which was filtered with a band-pass between 10 and 3000 Hz. The signals were integrated using a time constant of 100 ms. After cutting the central end of the nerve at the end of the experiment, the baseline noise of the signals was recorded, and this was subtracted from the

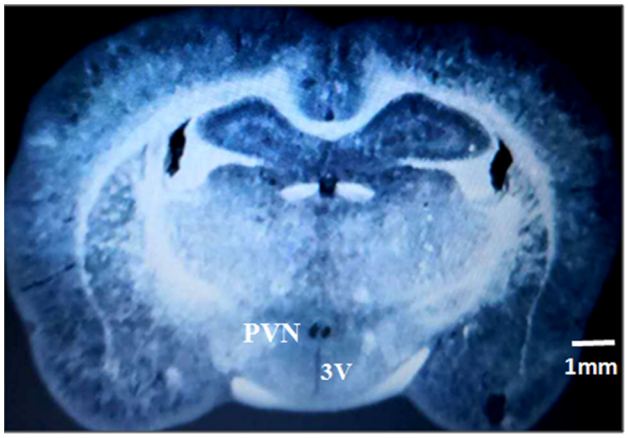

Fig. 1 A representative image of microinjection sites in the PVN evaluated by Evans blue diffusion. PVN, paraventricular nucleus; 3V, third ventricle.

integrated data. Baseline renal SNA and MAP were determined by averaging 1 min of their maximal responses after PVN microinjection, and the renal SNA change was expressed as the percentage change from baseline.

PVN Microinjection

To locate the PVN, each rat was fixed in a stereotaxic frame (Stoelting; Chicago, IL). The stereotaxic coordinates for the PVN were 1.8 mm caudal from bregma, 0.4 mm lateral to the midline, and 7.9 mm ventral to the dorsal surface. The bilateral microinjections were completed within 1 min, and a volume of 50 nL was injected through one glass micropipette (50 μm tip diameter) on each side of the PVN. For histological identification, the same volume of Evans Blue was injected into the sites at the end of each experiment. A representative image of microinjection sites in the PVN evaluated by Evans blue diffusion is shown in Fig. 1. If the microinjection sites were outside of the PVN, the data were excluded from analysis.

Table 2 Effects of IMD on metabolic parameters, anatomic data, and systolic blood pressure in OH rats after HFD consumption

Parameters	OH	OH + IMD
Body weight (g)	689 ± 51	581 ± 55*
Plasma glucose (mg/dL)	149 ± 14	131 ± 10
Plasma insulin (ng/mL)	3.1 ± 0.28	1.8 ± 0.12*
Plasma cholesterol (mg/dL)	60.7 ± 6.8	48.9 ± 5.8
Plasma triglyceride (mg/dL)	71.8 ± 8.5	63.4 ± 7.31
Heart weight (mg)	2298 ± 168	1765 ± 157*
Systolic blood pressure (mm Hg)	170 ± 16	138 ± 12*
Sum of WAT mass (g)	56.3 ± 5.1	38.7 ± 3.9*

Sum of WAT mass includes inguinal, retroperitoneal, epididymal and mesenteric WAT mass. Values are mean ± SEM. $n = 7$ for each group.

IMD intermedin, *OH* obesity-related hypertensive rats, *HFD* high-fat diet, *WAT* white adipose tissue

*$P < 0.05$ *vs* OH

Chemicals

IMD was from Bachem (Bubendorf, Switzerland), CGRP8-37 was from AnaSpec (Fremont, CA), and AM22-52 and the MAPK/ERK inhibitor U0126 were from Sigma Chemical (St. Louis, MO). The doses of IMD (50 pmol/L), AM22-52 (1 nmol/L), CGRP8-37 (0.2 nmol/L), U0126 (50 μmol/L), and LPS (0.5 μg/50 nL) were chosen with reference to our preliminary studies and published reports [25, 26]. The chemicals used for PVN microinjection were dissolved in normal saline or dimethylsulfoxide. IMD used for systemic micro-osmotic pump infusion was dissolved in saline.

Table 1 Metabolic parameters, anatomic data, and systolic blood pressure after HFD consumption

	Control	OB	OH
N	30	16	90
Body weight (g)	546 ± 11	653 ± 15*	651 ± 6*
Plasma glucose (mg/dL)	129 ± 7	141 ± 8	137 ± 9
Plasma insulin (ng/mL)	1.49 ± 0.07	2.93 ± 0.09*	2.91 ± 0.06*
Plasma cholesterol (mg/dL)	39.6 ± 0.8	59.2 ± 0.9*	58.9 ± 0.7*
Plasma triglyceride (mg/dL)	62.7 ± 0.9	88.9 ± 1.6*	89.3 ± 1.0*
heart weight (mg)	1669 ± 34	2197 ± 45*	2219 ± 29*
systolic blood pressure (mm Hg)	121 ± 8	130 ± 9	173 ± 6*,#
Sum of WAT mass (g)	25.9 ± 1.8	55.7 ± 3.9*	55.2 ± 1.2*

Values are mean ± SEM. Sum of WAT mass includes inguinal, retroperitoneal, epididymal and mesenteric WAT mass.

HFD high fat diet, *OB* obese and non-hypertensive rats, *OH* obesity-related hypertensive rats, *WAT* white adipose tissue

*$P < 0.05$ *vs* Control. #$P < 0.05$ *vs* OB

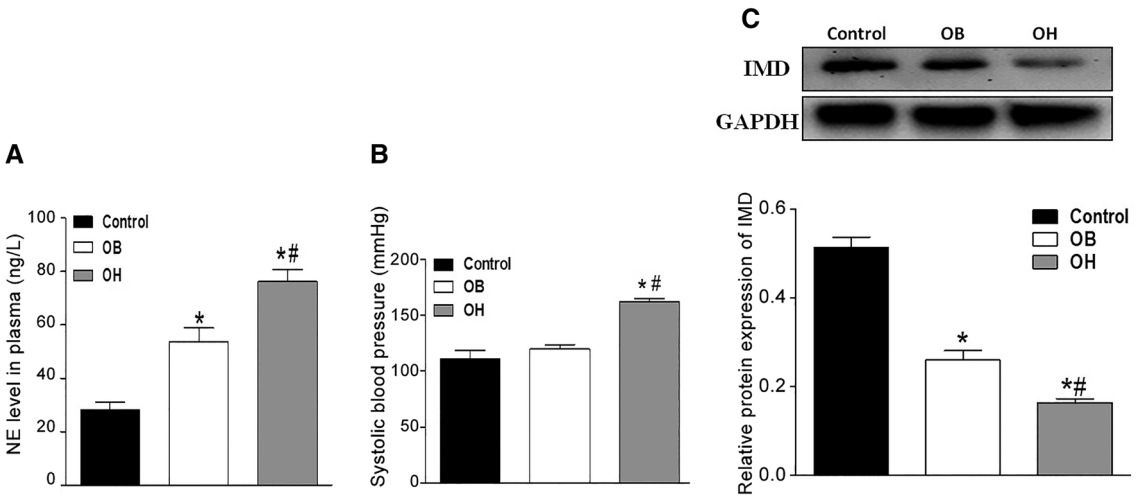

Fig. 2 Plasma norepinephrine (NE) (**A**, n = 6–8), systolic blood pressure (**B**, n = 6–8), and relative values of intermedin (IMD) in the paraventricular nucleus (**C**, n = 3–4) in control, obese (OB), and obesity-related hypertensive (OH) rats. Values are mean ± SEM. $^{*}P < 0.05$ *vs* control; $^{\#}P < 0.05$ *vs* OB.

Statistical Analysis

All data were analyzed using SPSS version 17.0 (SPSS Inc., Chicago, IL). Comparisons between two observations in each animal were analyzed using Student's t test. Differences between groups were determined with one-way ANOVA followed by the Bonferroni test for *post hoc* analysis of significance. All data are expressed as mean ± SEM. $P < 0.05$ was considered statistically significant.

Results

Metabolic and Anatomical Data

After HFD consumption for 12 weeks, plasma insulin, cholesterol, and triglycerides levels, as well as body weight, heart weight, and white adipose tissue mass were increased in the OB and OH rats (Table 1).

Effects of IMD Treatment on Metabolic and Anatomic Data in OH Rats

After HFD consumption for 12 weeks, OH rats were subcutaneously infused with saline (vehicle) or IMD (300 ng/kg per hour) by osmotic mini-pump and continued to be fed HFD for 4 weeks. The results showed that IMD decreased the plasma insulin level, heart weight, body weight, and white adipose tissue mass when compared with the OH group (Table 2). While the IMD treatment had a tendency to decrease the plasma glucose, total triglycerides, and cholesterol levels, the changes were not significant (Table 2).

Sympathetic Nerve Activity, Systolic Blood Pressure, and IMD Protein Expression in the PVN

The level of plasma NE is often used to evaluate basal SNA. When compared with the control group, a significant increase in NE levels (Fig. 2A) and a marked increase of SBP were found in OH rats (Fig. 2B). However, the IMD protein level in the PVN was clearly decreased in OB and OH rats. Moreover, the increased NE or decreased IMD levels were more marked in the OH rats.

Effects of Different Doses of IMD on Basal SNA

Acute microinjection of IMD into the PVN caused marked decreases in the renal SNA and MAP in control, OB, and OH rats (HFD for 12 weeks) in a dose-dependent manner (Fig. 3A–C), and this was more evident in OH rats (Fig. 3D).

TLR4 Protein Expression and the Effects of LPS on Basal SNA

The level of TLR4 protein in the PVN in OH rats was higher than that in OB or control rats. Acute microinjection of LPS (50 μg/50 nL) into the PVN caused greater increases in the renal SNA and MAP in OH rats than in OB or control rats (Fig. 4).

Effect of IMD on LPS-Induced Increase in Basal SNA

Recordings showed the effects of pretreatment of the PVN with saline or IMD on LPS-induced increases in basal SNA

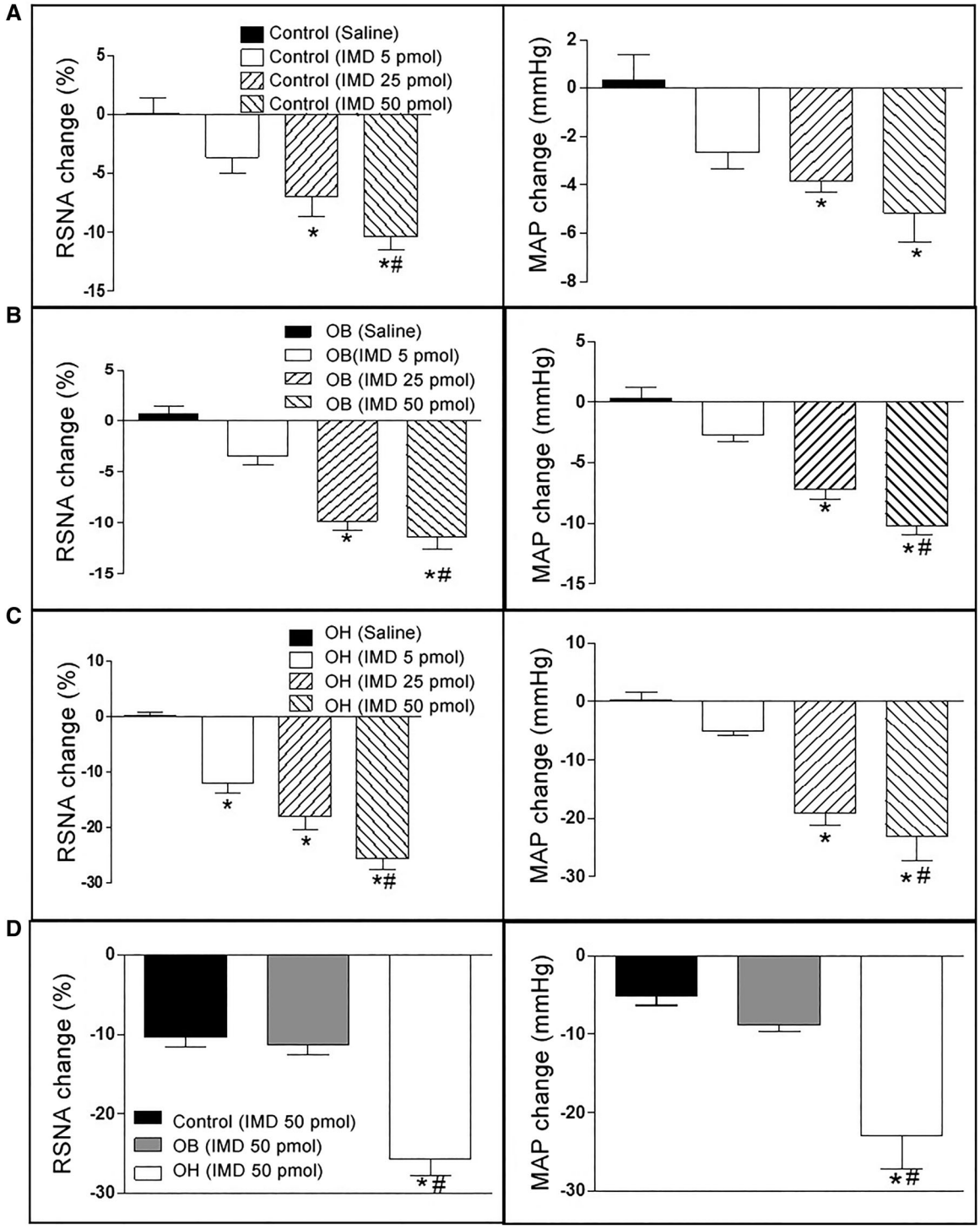

Fig. 3 Effects of paraventricular nucleus (PVN) microinjection of saline or 3 doses of intermedin (IMD, 5, 25, or 50 pmol) on the renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP) in control (**A**), obese (**B**), and obesity-related hypertensive (OH) rats (**C**), and a comparison of the effects of IMD (50 pmol) on RSNA and MAP in control, obese, and OH rats (**D**). Values are mean ± SEM. $^{*}P < 0.05$ *vs* saline; $^{\#}P < 0.05$ *vs* IMD 5 pmol (**A**–**C**); $^{*}P < 0.05$ *vs* control; $^{\#}P < 0.05$ *vs* OB (**D**); $n = 6$ for each group.

in OH rats (Fig. 5A). LPS (50 μg/50 nL) microinjection into the PVN significantly increased the renal SNA and MAP in OH rats compared with controls (Fig. 5B). The augmented response of the basal SNA to LPS was inhibited by the pretreatment with IMD (50 pmol) in both control and OH rats (Fig. 5B).

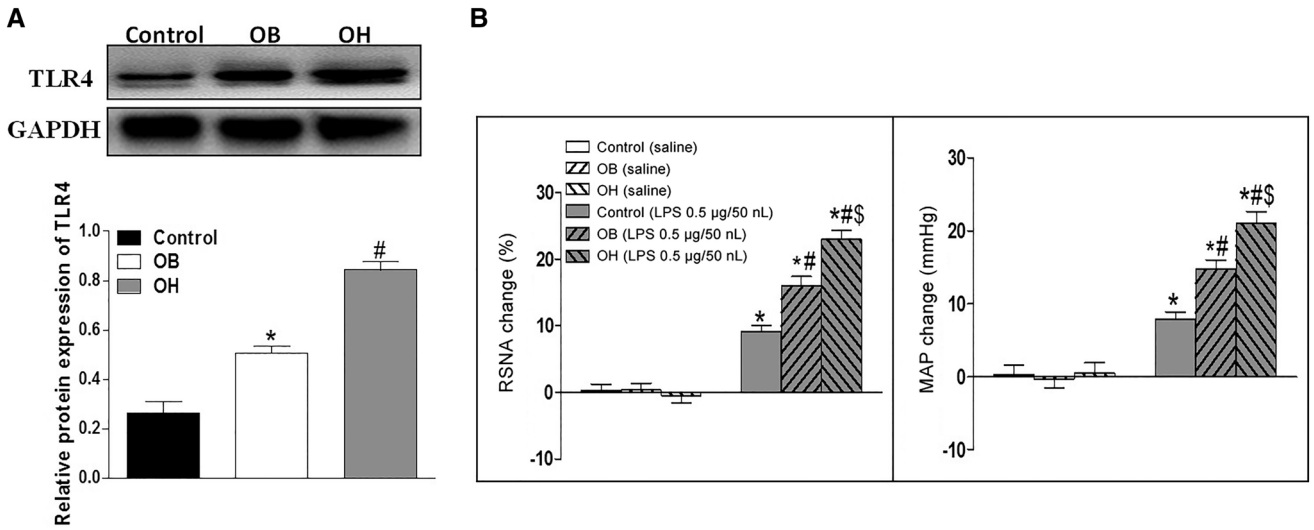

Fig. 4 Relative levels of Toll-like receptor 4 (TLR4) protein in the paraventricular nucleus (PVN) (**A**) and effects of PVN microinjection of saline or LPS (0.5 μg/50 nL) on renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP) in control, obese (OB), and obesity-related hypertensive (OH) rats (**B**). Values are mean ± SEM. $^{*}P < 0.05$ *vs* control; $^{\#}P < 0.05$ *vs* OB (**A**, $n = 3$–4); $^{*}P < 0.05$ *vs* saline; $^{\#}P < 0.05$ *vs* control; $^{\$}P < 0.05$ *vs* OB (**B**); $n = 6$ for each group.

Effects of IMD on LPS-Stimulated MAPK Activation

To elucidate the signaling mechanism underlying the effect of sympathetic suppression of the LPS-induced increase in basal SNA by IMD, we assessed the activation of the p38, ERK1/2, and JNK MAPKs in OH rats. We found that LPS treatment led to the activation of all three MAPKs. Pretreatment with IMD markedly attenuated the ERK1/2 MAPK phosphorylation stimulated by LPS (Fig. 6C), but had no significant effect on that of p38 and JNK (Fig. 6A, B).

Effects of ERK Inhibitor on LPS-Induced Increase in Basal SNA, and Receptor Antagonist CGRP8-37 and AM22-52 on IMD-Induced Decrease in Basal SNA

Microinjection of the ERK inhibitor U0126 into the PVN significantly decreased the renal SNA and MAP (Fig. 7A), whereas the AM receptor antagonist AM22-52 clearly increased them in OH rats. Microinjection of the CGRP receptor antagonist CGRP8-37 into the PVN had no significant effect on the renal SNA and MAP in OH rats (Fig. 7B). The sympathoexcitatory and pressor roles of LPS were effectively inhibited by pretreatment with the ERK inhibitor in the PVN (Fig. 7A). Moreover, the sympathoinhibitory and depressor effects of IMD were significantly attenuated by pretreatment with AM22-52 but not CGRP8-37 in the PVN in OH rats (Fig. 7B).

Impact of Receptor Antagonist AM22-52 Pretreatment in the PVN on the Effects of the IMD Response to LPS-Induced SNA and ERK Activation

To determine whether endogenous AM receptors in the PVN modulate the IMD responses to LPS-induced sympathoexcitation and ERK activation in OH rats, we microinjected the AM receptor antagonist AM22-52 into the PVN. As shown in Fig. 8, blockade of endogenous AM receptors effectively inhibited the sympathoinhibitory and depressor effects of IMD responses to LPS-induced sympathetic overactivity, and it also significantly attenuated the inhibitory effect of IMD on the ERK activation caused by LPS.

Effects of Chronic Systemic Treatment with IMD on Basal SNA and BP, LPS-Induced Sympathoexcitation, and TLR4 Protein Expression and ERK Activation

Alzet osmotic minipumps (model 2004, Durect Corp.) containing IMD (300 ng/kg per hour) or saline were implanted subcutaneously into the OH rats for infusion, and they were fed an HFD for 28 days. We found that chronic systemic treatment with IMD lowered the SNA and BP (Fig. 9A, B), attenuated LPS-induced sympathoexcitation (Fig. 9C), and decreased TLR4 protein expression and ERK activation in the PVN (Fig. 9D, E).

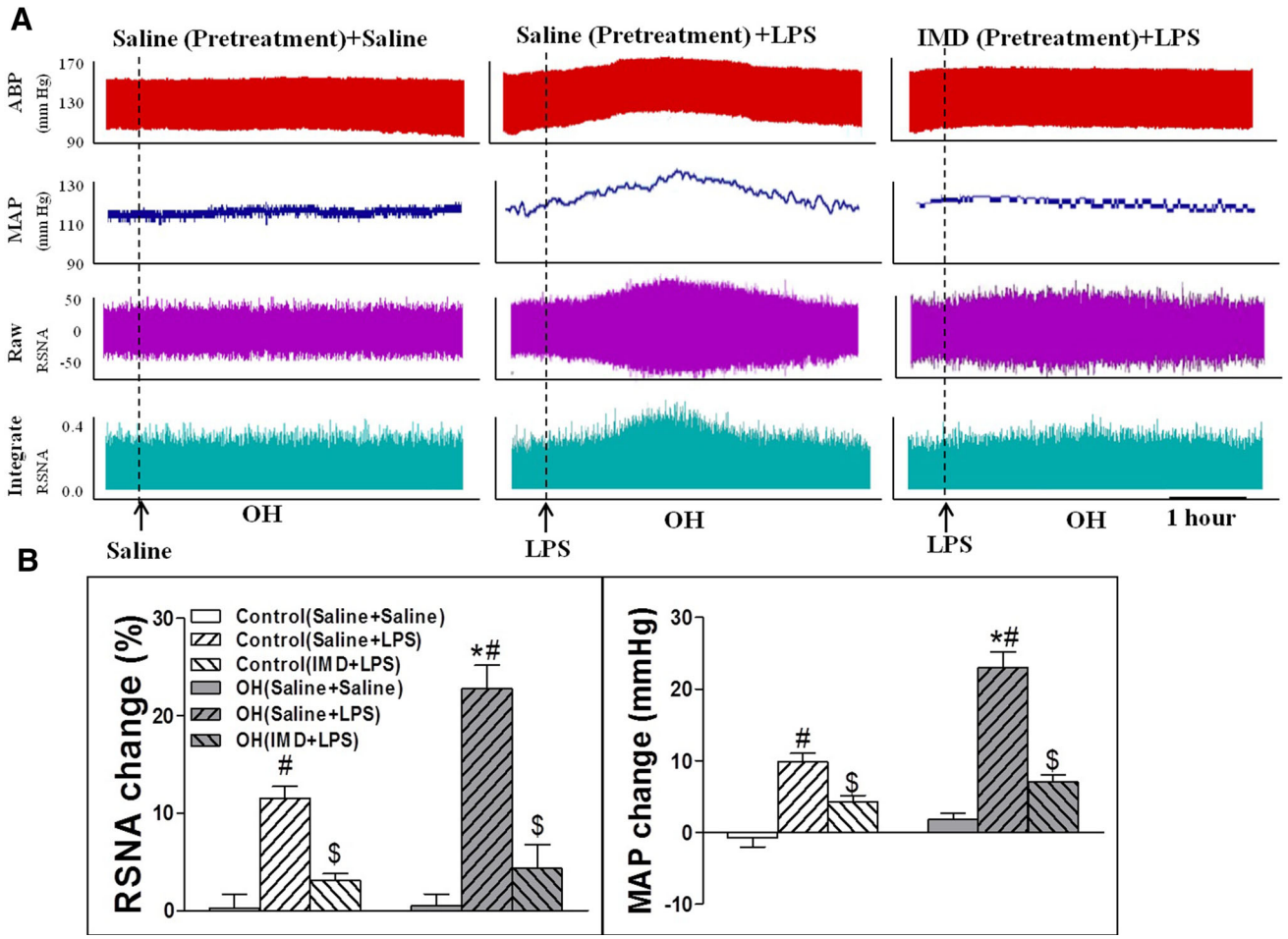

Fig. 5 **A** Representative recordings showing the effects of saline or IMD (50 pmol) pretreatment of the paraventricular nucleus (PVN) on renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP) responses to LPS (0.5 μg/50 nL) in the PVN in obesity-related hypertensive (OH) rats. **B** Statistical analysis of data as in **A**. LPS was administered 10 min after the pretreatment. Values are mean ± SEM. $^{*}P < 0.05$ *vs* control; $^{\#}P < 0.05$ *vs* saline + saline, $^{\$}P < 0.05$ *vs* saline + LPS; $n = 6$ for each group.

Discussion

In the present study, the OH rats had a higher plasma NE level (an indirect indicator of SNA), indicating enhanced sympathetic outflow compared to control rats. Activation of TLR4 in the PVN leads to increased sympathetic activation in OH rats. However, a novel finding of this study was that microinjection of IMD into the PVN resulted in a dramatic reduction in SNA and MAP, and chronic systemic infusion of IMD also caused obvious clear decreases in the plasma level of NE and SBP in the OH rats, suggesting that IMD in the PVN or after peripheral application plays a key role in the inhibition of sympathoexcitation in OH rats. Furthermore, our results also showed the roles and the possible mechanisms of IMD in LPS-induced sympathoexcitatory and pressor responses.

Excessive sympathetic activity contributes to the pathogenesis of OH and the progression of organ damage, and intervention in the sympathetic activation has been considered to be an important strategy for attenuating hypertension and related structural alterations in organs [27, 28]. In the present study, the IMD content of the PVN was downregulated in OH rats, and microinjection of IMD into the PVN caused greater decreases in the renal SNA and MAP in OH rats than in control rats, indicating that the lower IMD expression in the PVN may be important in the pathogenesis of sympathetic activation in OH. Increasing IMD expression in the PVN may be beneficial for the attenuation of hypertension in OH rats.

At present, no unique receptor for IMD has been identified. IMD shares the receptor system consisting of calcitonin receptor–like receptor (CRLR) and receptor activity-modifying proteins (RAMP1, 2, 3) with AM and CGRP. The CRLR/RAMP2 or CRLR/RAMP3 complex forms the AM receptor, whereas CRLR/RAMP1 forms the CGRP receptor. IMD can bind nonselectively to all three complexes [15]. In the present study, the sympathoinhibitory and depressor effects of IMD were significantly

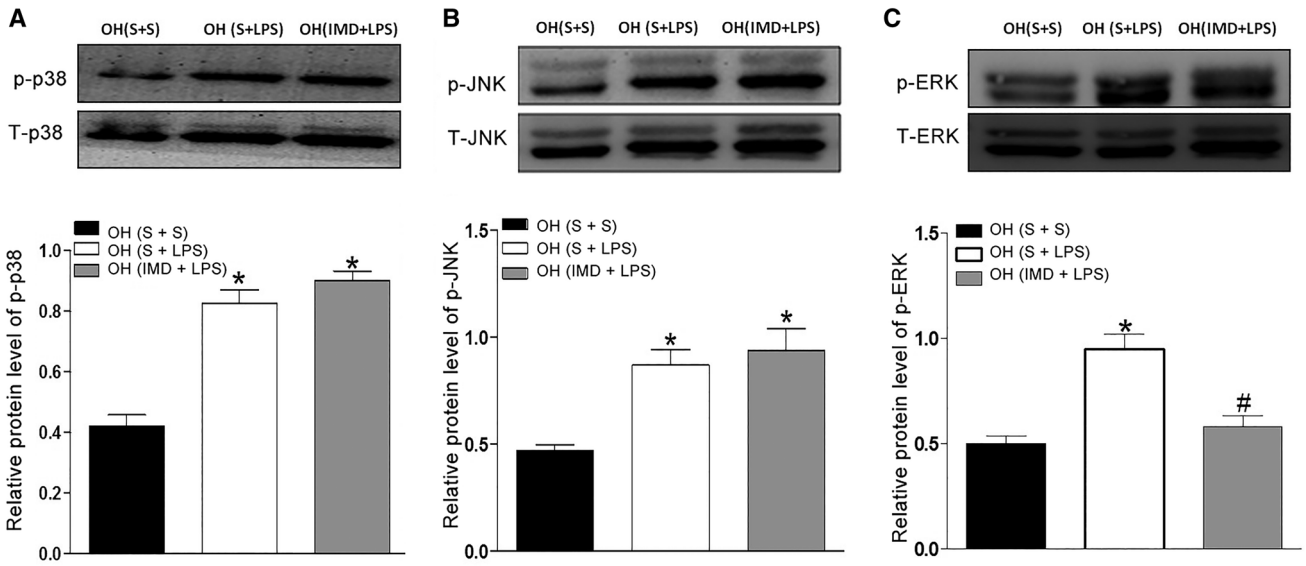

Fig. 6 Effect of paraventricular nucleus (PVN) pretreatment with saline or IMD (50 pmol) on the LPS (0.5 μg/50 nL)-induced phosphorylated and total MAP kinases (MAPKs). **A**–**C** Activation of p38 (**A**), JNK (**B**), and ERK (**C**) MAPKs in the PVN of obesity-related hypertensive (OH) rats. S, Saline. Values are mean ± SEM. $^{*}P < 0.05$ *vs* S + S; $^{\#}P < 0.05$ *vs* S + LPS; n = 3–4 for each group.

attenuated by pretreatment with the AM receptor antagonist AM22-52 but not the CGRP receptor antagonist CGRP8-37 in the PVN in OH rats. The results suggest that AM receptors rather than CGRP receptors mainly mediate the effects of IMD in the PVN on SNA in OH rats. This may depend on the amount of protein expression of RAMP1,2,3, which needs to be investigated in a future study.

IMD is involved in the regulation of the central and peripheral circulation and water-electrolyte homeostasis [29, 30]. Previous studies have demonstrated that IMD protects mice from atherosclerosis, reduces insulin resistance in HFD-induced obese mice [31], inhibits chronic inflammation in adipose tissue, and improves the systemic insulin sensitivity of mice with hyperhomocysteinemia [23]. Decreased IMD content has been reported in the plasma of diabetic rats [32], and intermedin attenuates LPS-induced inflammation in the rat testis [33]. More importantly, recently published data from our group have shown that IMD in the PVN inhibits SNA in rats with hypertension or chronic heart failure [25, 34]. These studies suggest that IMD may act against the sympathoexcitatory effects induced by LPS in the PVN in HFD-induced OH rats.

We also investigated the effects of PVN microinjection of the TLR4 agonist LPS in OH rats, and explored whether IMD plays a role in the LPS-induced sympathoexcitatory and pressor responses. We found a significant increase in TLR4 protein expression in the PVN of OH rats, indicating that TLR4 upregulation in the PVN could be one of the characteristics of the hypertensive response in these rats. LPS application into the PVN caused evident enhancement of SNA and MAP. This is the first demonstration that increased TLR4 expression in the PVN contributes to sympathoexcitatory and hypertensive responses in OH rats. IMD pretreatment in the PVN prevented, at least in part, the increases in SNA and BP, and attenuated the MAPK/ERK activation induced by LPS. However, the action of IMD was effectively blocked by the AM receptor antagonist AM22-52 in the PVN. In addition, an ERK inhibitor partially inhibited LPS-induced sympathetic activation. These results reveal that IMD, *via* AM receptors, partially attenuates TLR4-mediatd sympathoexcitation by inhibiting ERK activation.

In recent years, the effects of neural inflammation on systemic cardiovascular changes have been explored [35]. MAPK/ERK activation is an integral part of the TLR4 signaling involved in inflammation [36]. This activation is involved in sympathoexcitation [37]. For instance, angiotensin II-mediated MAPK/ERK activation modulates inflammation, and increases presynaptic glutamate release in the brain [38, 39]. LPS stimulates TLR4 on cells and activates MAPK kinases, leading to the phosphorylation of p38 MAPK, ERK1/2, and JNK. We found the same results in the PVN after LPS microinjection, and IMD inhibited ERK activation, but not p38 and JNK activation. Moreover, U0126 (a selective ERK1/2 inhibitor) pretreatment in the PVN partially inhibited the effects of LPS on SNA and BP. These results suggested that IMD in the PVN partially attenuates LPS-induced sympathoexcitation *via* the inhibition of ERK activation. In the inflammatory pathways, sympathoexcitation caused by LPS may involve alteration

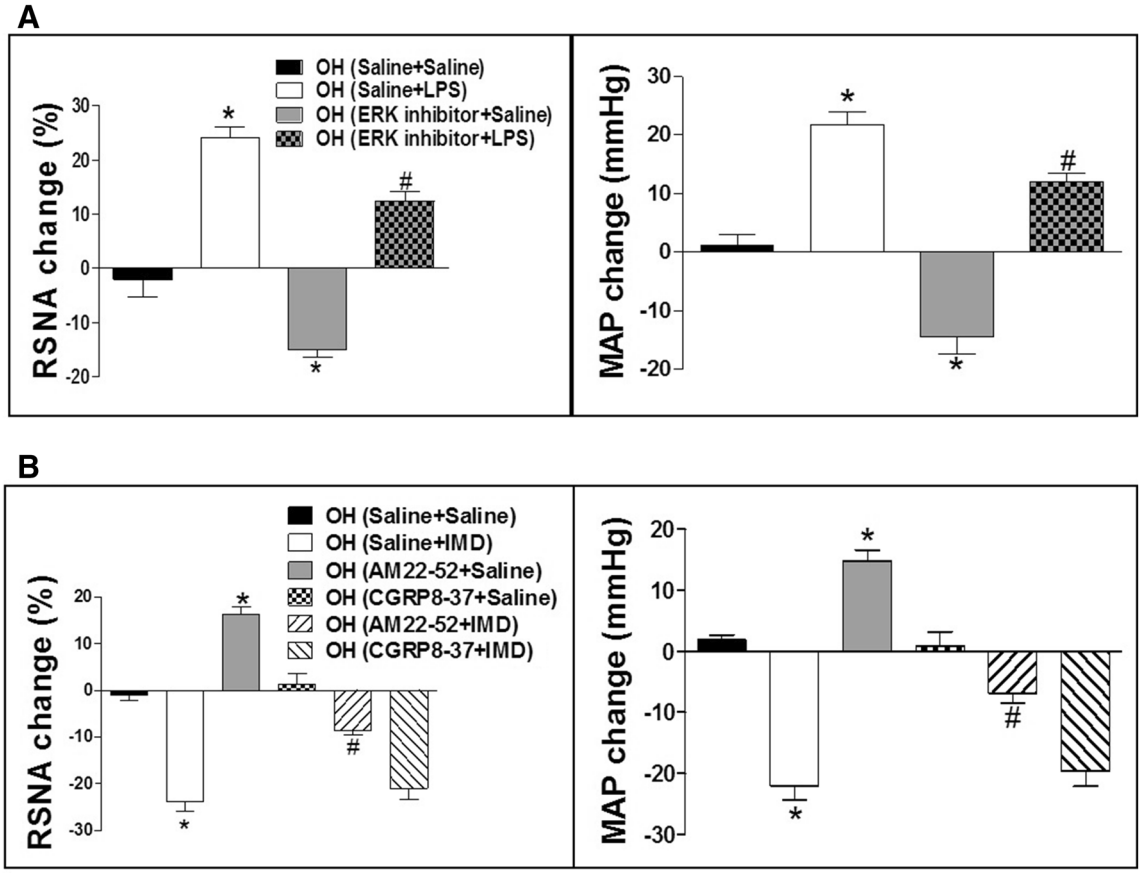

Fig. 7 Effects of paraventricular nucleus (PVN) pretreatment with saline, the ERK inhibitor U0126 (50 μmol/L) (**A**), or the AM receptor antagonist AM22-52 and CGRP receptor antagonist CGRP8-37 (**B**) on the renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP) responses to LPS (0.5 μg/50 nL) or IMD (50 pmol) in the PVN in obesity-related hypertensive (OH) rats. Values are mean ± SEM. $^{*}P < 0.05$ *vs* saline + saline; $^{\#}P < 0.05$ *vs* saline + LPS (**A**); $^{*}P < 0.05$ *vs* saline + saline; $^{\#}P < 0.05$ *vs* saline + IMD (**B**); $n = 6$ for each group.

of the excitatory (glutamate and catecholamines) and inhibitory (GABA) neurotransmitters in the PVN [26]. Therefore, inhibition of MAPK/ERK activation in the PVN may reduce the severity of sympathoexcitation in OH rats. The outcome of this study was that PVN TLR4 activation increased PVN MAPK/ERK phosphorylation, and this was inhibited by IMD application. Moreover, AM22-52 pretreatment effectively reversed the IMD effects in OH rats.

In the present study, chronic IMD administration resulted in significant reduction in the plasma NE level and BP in OH rats. More importantly, IMD lowered PVN TLR4 protein expression and ERK activation, and inhibited LPS-induced sympathetic overactivity. These results provide mechanistic evidence that the detrimental effects seen in obesity-related hypertension are mediated, at least in part, by TLR4 in the PVN and that inhibition of TLR4 downstream ERK signaling by IMD attenuates sympathoexcitatory and hypertensive responses in the PVN. Our results suggest TLR4 as a new therapeutic target for IMD, exerting effective control of sympathetic overactivity and blood pressure in OH rats.

In contrast to the effects of peripheral infusion of IMD, intracerebroventricular administration of IMD raises BP [40], and microinjection of IMD into the nucleus tractus solitarii (NTS) increases SNA in normal rats [41]. However, IMD administered to the PVN decreases the SNA in normal and disease model rats [25], indicating that IMD has different functions in different encephalic regions. Presympathetic PVN neurons receive afferent projections from the NTS, and presympathetic efferent projections from the PVN target the rostral ventrolateral medulla (RVLM), NTS, and sympathetic preganglionic neurons in the spinal cord. We have demonstrated that an RVLM lesion almost abolishes the effects of IMD in the NTS and in the PVN. A PVN lesion only attenuates the actions of IMD in the NTS, and NTS lesions do not affect the actions of IMD in the PVN. Microinjection of IMD into the NTS neutralizes the effects of IMD in the PVN [25]. These results indicate that both the PVN and RVLM promote the

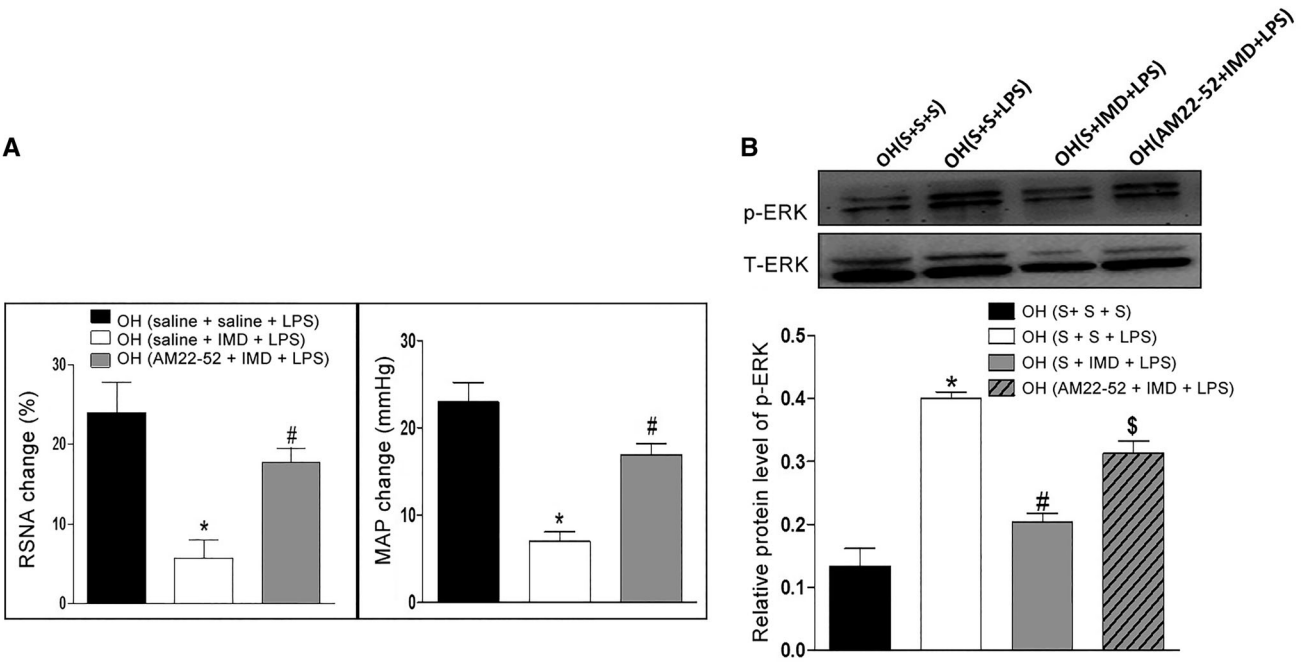

Fig. 8 Effects of paraventricular nucleus (PVN) pretreatment with saline or the AM receptor antagonist AM22-52 on IMD responses to LPS (0.5 μg/50 nL)-induced changes of renal sympathetic nerve activity (RSNA), mean arterial pressure (MAP) and ERK activation in the PVN in obesity-related hypertensive (OH) rats. Values are mean ± SEM. $^{*}P < 0.05$ *vs* S + S + LPS; $^{\#}P < 0.05$ *vs* S + IMD + LPS (**A**). $n = 6$ for each group. $^{*}P < 0.05$ *vs* S + S + S; $^{\#}P < 0.05$ *vs* S + S + LPS; $^{\$}P < 0.05$ *vs* S + IMD + LPS (**B**). $n = 3$–4 for each group. S: Saline.

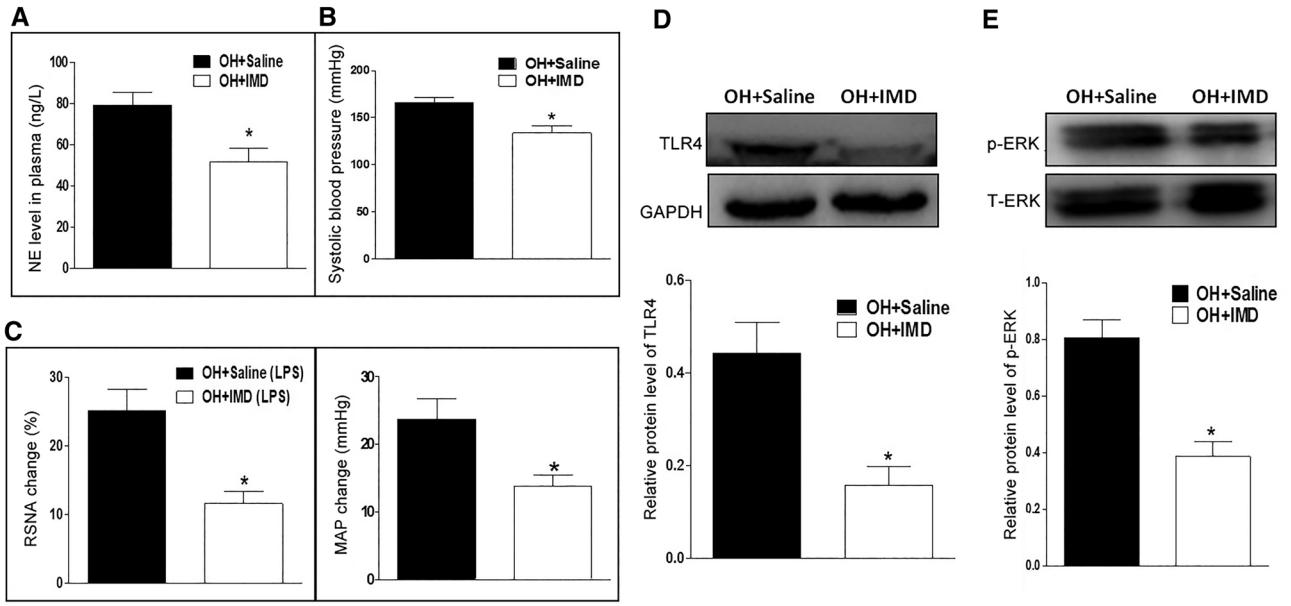

Fig. 9 Effects of chronic systemic application of intermedin (IMD, 300 ng/kg per hour) for 28 days on the plasma NE level (**A**), systolic blood pressure (**B**), and PVN TLR4 protein expression and ERK activation (**D**, **E**. $n = 3$–4), and the renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP) responses to LPS (0.5 μg/50 nL) in the PVN (**C**) in obesity-related hypertensive (OH) rats. Values are mean ± SEM. $^{*}P < 0.05$ *vs* OH + saline. $n = 6$–8 for (**A**–**C**).

integration of the sympathoinhibitory and depressor activity of IMD in the PVN and the sympathoexcitatory and pressor activity in the NTS. In the present study, IMD in the PVN attenuated SNA and LPS-induced sympathoexcitation in OH rats, suggesting that the PVN may be one of the main sites of action of IMD for inhibiting SNA in OH.

Taken together, our data suggest that PVN TLR4 and ERK activation are major events in the LPS-induced enhancement of SNA in OH rats; IMD in the PVN decreases SNA and BP *via* activating the AM receptor pathway, and this is partly due to the inhibition of LPS-induced ERK phosphorylation, leading to decreased sympathetic activation in OH rats. Based on our results, IMD might be considered as a future target for obesity-related hypertension treatment.

Acknowledgements We gratefully acknowledge the generous support of the Collaborative Innovation Center for Cardiovascular Disease Translational Medicine. This work was supported by the National Natural Science Foundation of China (81000106 and 81470539).

Compliance with ethical standards

Conflict of interest The authors claim that there are no conflicts of interest.

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