Neurosci. Bull. February, 2019, 35(1):57–66
https://doi.org/10.1007/s12264-018-0308-5

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

ORIGINAL ARTICLE

Chronic Intracerebroventricular Infusion of Metformin Inhibits Salt-Sensitive Hypertension *via* Attenuation of Oxidative Stress and Neurohormonal Excitation in Rat Paraventricular Nucleus

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Received: 11 April 2018 / Accepted: 26 September 2018 / Published online: 14 November 2018

Abstract Metformin (MET), an antidiabetic agent, also has antioxidative effects in metabolic-related hypertension. This study was designed to determine whether MET has anti-hypertensive effects in salt-sensitive hypertensive rats by inhibiting oxidative stress in the hypothalamic paraventricular nucleus (PVN). Salt-sensitive rats received a high-salt (HS) diet to induce hypertension, or a normal-salt (NS) diet as control. At the same time, they received intracerebroventricular (ICV) infusion of MET or vehicle for 6 weeks. We found that HS rats had higher oxidative stress levels and mean arterial pressure (MAP) than NS rats. ICV infusion of MET attenuated MAP and reduced plasma norepinephrine levels in HS rats. It also decreased reactive oxygen species and the expression of subunits of NAD(P)H oxidase, improved the superoxide dismutase activity, reduced components of the renin-angiotensin system, and altered neurotransmitters in the PVN. Our findings suggest that central MET administration lowers MAP in salt-sensitive hypertension *via* attenuating oxidative stress, inhibiting the renin-angiotensin system, and restoring the balance between excitatory and inhibitory neurotransmitters in the PVN.

Keywords Hypertension · Paraventricular nucleus · Sympathoexcitation · Metformin · Oxidative stress

Introduction

Activation of the sympathetic nervous system is one of the major reasons for the occurrence and development of hypertension [1]. The hypothalamic paraventricular nucleus (PVN) is responsible for sympathetic drive and cardiovascular control [2]. The PVN controls the level of sympathetic outflow mainly by the integration of neurohumoral activity [3]. As potent intracellular second

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messengers, reactive oxygen species (ROS), especially superoxide anion, mediate the signaling pathways causing hypertension [4]. It has been established that NAD(P)H oxidase plays a major role in ROS production during the development of hypertension [5]. In addition, angiotensin II (ANG II)-induced hypertension has been linked to the promotion of ROS formation in the PVN [6, 7]. Therefore, reduction of oxidative stress is important in the prevention and treatment of hypertension. The renin-angiotensin system (RAS) is an important mediator that contributes to cardiovascular diseases [8]. As the main bioactive component of the RAS, ANG II acts in the central nervous system *via* binding to the ANG II type 1 receptor (AT1-R), whereby it contributes to sympathoexcitation and the hypertensive response [9]. Neurotransmitters in the PVN such as glutamate, norepinephrine (NE), and gamma-aminobutyric acid (GABA) are involved in the development of hypertension [10]. NE and glutamate are vital excitatory neurotransmitters, while GABA is a major inhibitory neurotransmitter in the PVN [11]. Many studies have indicated that sympathoexcitation and hypertension are due to high levels of excitatory neurotransmitters and low levels of inhibitory neurotransmitters in the PVN [12]. Therefore, ROS, the RAS, and neurotransmitters in the PVN are all involved in the pathogenesis of hypertension.

Metformin (MET), the oldest and most widely used glucose-lowering drug, is likely to also be effective in the prevention of cardiac and vascular disease [13], having been shown to reduce oxidative stress levels in patients [14, 15]. In addition, Tain *et al*. [16] have reported that prenatal MET therapy in rats prevents the hypertension of developmental origin induced by a maternal high-fructose plus a high-fat diet *via* the regulation of nutrient-sensing signals, uric acid, oxidative stress, and the nitric oxide pathway [16]. Importantly, MET has been reported to markedly decrease blood pressure in rats [17, 18]. Both peripheral and intracerebroventricular MET administrations decrease blood pressure in hypertensive rats, due to its inhibition of sympathetic activity [19, 20]. But the specific mechanisms are unclear. Considerable evidence has shown that MET can cross the blood-brain barrier to accumulate in the hypothalamus and directly affect the central nervous system [21, 22]. Thus we hypothesized that the antihypertensive effect of MET may be associated with the regulation of central sympathetic outflow and neuroendocrine responses. In this study, we investigated the protective action of MET against salt-sensitive hypertension and determined if this was attributable to reduced oxidative stress and sympathetic activity in the PVN. We also determined the involvement of the RAS and neurotransmitters in the effect of MET.

Methods

Ethics Statement

All procedures involving animals were approved by the Animal Care and Use Committee of Xi'an Jiaotong University (Xi'an, China) and performed according to the Guidelines for the Care and Use of Experimental Animals of the United States National Institutes of Health.

Animals and Experimental Protocols

Eight-week-old male Dahl salt-sensitive rats from the laboratory of Professor Jian-Jun Mu (Department of Cardiology, The First Affiliated Hospital of Xi'an Jiaotong University) were housed in a room with a 12-h light/dark cycle and temperature and humidity control. They were allowed access to standard chow and tap water *ad libitum*. They were fed for 6 weeks with a high-salt diet (HS, 8% NaCl) or a normal-salt diet (NS, 0.3% NaCl). All rats were anesthetized by intraperitoneal (i.p.) injection of a ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture. The animals were placed in a stereotaxic frame, and the skull was leveled between bregma and lambda. A minipump (Alzet Model 2006, Durect Corp., Cupertino, CA) was placed subcutaneously on the back of each rat. The coordinates used for intracerebroventricular (ICV) cannulation were 0.5 mm posterior to bregma, 1.5 mm lateral to the midline, and 2.7 mm below the skull surface [23]. MET (25 μg/day) or vehicle (artificial cerebrospinal fluid) was continuously infused ICV for 6 weeks [24]. At the end of the experiment, rats were anesthetized with i.p. injection of a ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture and euthanized by decapitation in order to collect blood and brain tissue for immunological and molecular biological assessment.

Mean Arterial Pressure Measurement

Blood pressure and heart rate (HR) were determined by tail-cuff occlusion using an acute method as previously described [25, 26]. Arterial pressure was measured noninvasively *via* a tail-cuff and its recording system (BP100A, 113 Chengdu Techman Software Co., Ltd, China). Unanesthetized rats were warmed to an ambient temperature of 30°C by placing them in a holding device mounted on a thermostatically-controlled warming plate. All animals were habituated to the blood pressure system and to the holders daily for one week prior to the initiation of experimental measurements. Each rat was allowed to adapt to the cuff for 10 min before measurement. Blood pressure

values were averaged from six consecutive cycles per day from each rat.

At the end of week 10, the rats were anesthetized by i.p. injection of a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture. A polyethylene catheter was inserted into the carotid artery to measure mean arterial pressure (MAP) and HR. The catheter was pre-filled with 0.1 mL heparinized saline (50 units/mL) and connected to a pressure transducer attached to a digital BP monitor and polygraph (BL420, Chengdu Techman Software Co. Ltd, China). MAP and HR data were collected for 30 min and averaged.

Biochemical Assays

Plasma NE levels were measured using ELISA kits (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. SOD activity in the PVN was assessed using an ELISA kit (Nanjing Jiancheng BioEngineering Institute, Nanjing, China) following the manufacturer's instructions. The standards or sample diluents were added and incubated in wells of a microtiter plate pre-coated with a specific antibody. Conjugate was added and incubated for 1 h at 37°C and then washed. The reactions were stopped with stop solution, and read at 405 nm for NE and 450 nm for SOD using a microtiter plate reader (MK3, Thermo Fisher Scientific, Waltham, MA) [27].

High-Performance Liquid Chromatography (HPLC)

The levels of NE, glutamate, and GABA in the PVN were measured using HPLC with electrochemical detection (Waters-2465, Waters Corp., Milford, MA) as previously described [11, 28, 29]. Briefly, samples or standards were derivatized with o-phtaldialdehyde; 20 μL of the resulting mixture was automatically loaded onto a Novapark C18 reverse-phase column (150 mm × 4.6 mm, 4 μm particle size, Waters), using a refrigerated autoinjector. The mobile phase consisted of 0.05 mol/L NaH_2PO_4 (pH 6.8) with 20% methanol, and the flow rate was 1 mL/min delivered by a Waters pump. The concentrations of NE, glutamate and GABA were detected and analyzed using Empower 3 analytical software (Waters).

Real-Time Polymerase Chain Reaction

Total RNA was isolated using RNeasy kits (Qiagen, Duesseldorf, Germany) according to the manufacturer's instructions, and 1 μg of purified RNA was reverse transcribed with a high-capacity cDNA reverse transcription kit (Bio-Rad Laboratories, Inc., Hercules, CA). The mRNA levels were analyzed by quantitative real-time PCR using specific primers. The primers for NADPH oxidase (NOX)-2, NOX-4, and glyceraldehyde-phosphate dehydrogenase (GAPDH) were as follows: NOX-2 Forward 5′-CTGCCAGTGTGTCGGAATCT-3′, Reverse 5′-TGTGAATGGCCGTGTGAAGT-3′; NOX-4 Forward 5′-GGATCACAGAAGGTCCCTAGC-3′, Reverse 5′-AGAAGTTCAGGGCGTTCACC-3′; GAPDH Forward 5′-AGACAGCCGCATCTTCTTGT-3′, Reverse 5′-CTTGCCGTGGGTAGAGTCAT-3′. The quantitative fold changes in mRNA expression were determined relative to GAPDH mRNA levels in each group [7].

Immunofluorescence and Immunohistochemistry

Rats were anesthetized with a ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture (i.p.) and transcardially perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde. Samples were fixed overnight in 4% paraformaldehyde at 4°C, and then immersed in 30% sucrose for at least 2 days. Samples were embedded in OCT and cut into several 14-μm transverse sections, about 21.80 mm from bregma, on a sliding microtome; sections were mounted on slides and stored at − 80°C.

Sections were then washed in PBS for 20 min, permeabilized in 0.2% Triton in Tris-buffered saline for 1 h, blocked using 5% normal goat serum with 0.2% Triton in Tris-buffered saline for 1 h, and incubated with primary antibody in blocking buffer at 4°C overnight. The primary antibodies used were: anti-NOX-2 (1: 300, sc-20782, Santa Cruz Biotechnology, Dallas, TX), anti-angiotensin-converting-enzyme (ACE, 1:200, bs-0439R, Biosynthesis Biotechnology, Beijing, China), and anti-glutamate decarboxylase 67 (GAD67, 1:300, sc-7512, Santa Cruz Biotechnology). After washing in PBS, sections were further incubated with biotinylated secondary antibodies (at 1:300 dilution, ABC staining system kit, Santa Cruz, CA), Alexa 594-labeled anti-mouse secondary antibody (1:200, red fluorescence) (Invitrogen, Carlsbad, CA) for 60 min at room temperature [30].

Superoxide generation in the PVN was determined using fluorescence-labeled dihydroethidium, as previously described [31]. All sections were imaged on a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan).

Western Blotting Analysis

The PVN tissue was homogenized in lysis buffer and Western blotting was performed as previously described [28, 32]. The protein concentration was measured, loaded onto a SDS-PAGE gel, and transferred to a polyvinylidene fluoride membrane. The membrane was then incubated overnight at 4°C with the primary antibodies anti-NOX-4 (1:200, sc-21860, Santa Cruz Biotechnology), anti-SOD (1:300, FL-154, Santa Cruz Biotechnology), anti-AT1-R

(1:300, sc-579, Santa Cruz Biotechnology), anti-tyrosine hydroxylase (TH; 1:300, sc-14007, Santa Cruz Biotechnology), anti-GAD67 (1:300, sc-7512, Santa Cruz Biotechnology), and anti-β-actin (1:500, Thermo Scientific). After four washes with wash buffer for 10 min each, blots were incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:5,000, Santa Cruz Biotechnology). Protein loading was controlled by probing all blots with β-actin antibody (Thermo Scientific) and normalizing their protein intensities to that of β-actin. Band densities were analyzed with NIH ImageJ software.

Statistical Analysis

All data are presented as mean ± SEM and $P < 0.05$ was considered statistically significant. Statistical analyses were performed using Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA). MAP was analyzed by repeated measures ANOVA. One-way ANOVA with Tukey's *post hoc* test was applied to analyze protein levels in the PVN, plasma NE, numbers of positive neurons, fluorescence intensity, and western blotting data. Two-way ANOVA followed by Bonferroni's *post hoc* was used to analyze cardiovascular and autonomic parameters (MAP and HR) after ICV infusion of vehicle or MET.

Results

MET Decreases Blood Pressure in Hypertensive Rats

The HS diet elicited sustained elevation of MAP and HR compared with the NS group. Chronic ICV infusion of MET significantly attenuated the salt-induced increase in MAP in hypertensive rats, but not in the NS diet groups. However, there were no significant infusion-related changes in HR in the NS and HS groups (Fig. 1A and Table 1).

MET Reduces Plasma Norepinephrine Levels in Hypertensive Rats

Salt-induced hypertensive rats had significantly higher levels of plasma NE (Fig. 1B) than those on an NS diet. ICV infusion of MET reduced the levels of plasma NE (Fig. 1B) in the hypertensive rats.

MET Attenuates Oxidative Stress in the PVN of Hypertensive Rats

The HS diet induced significantly higher levels of NOX-2 immunoreactivity (Fig. 2A, B), NOX-4 protein expression, and fluorescence-labelled dihydroethidium (Figs. 2C and 4A, B) than the NS diet. ICV infusion of MET attenuated these changes in hypertensive rats (Figs. 2 and 4A, B). In addition, the NOX-2 (Fig. 3A) and NOX-4 (Fig. 3B) mRNA expression was significantly higher in HS rats than in NS rats. ICV infusion of MET decreased the NOX-2 and NOX-4 mRNA expression in hypertensive rats (Fig. 3A, B). Furthermore, the decreased SOD protein expression

Table 1 Effects of ICV infusion of vehicle or MET on MAP and HR in rats on an NS or a HS diet ($n = 7$).

Group	MAP (mmHg)	HR (beats/min)
NS+ICV vehicle	102.2 ± 6.5	372.2 ± 17.8
NS+ICV MET	99.6 ± 5.6	366.8 ± 18.3
HS+ICV vehicle	156.5 ± 10.1*	430.3 ± 19.6*
HS+ICV MET	124.7 ± 9.7*†	417.9 ± 22.1*

ICV, intracerebroventricular; MET, metformin; MAP, mean arterial pressure; HR, heart rate; NS, normal-salt; HS, high-salt. Values are mean ± SEM. *$P < 0.05$ *vs* NS rats (NS + ICV vehicle or NS + ICV MET); †$P < 0.05$ HS + ICV vehicle *vs* HS + ICV MET.

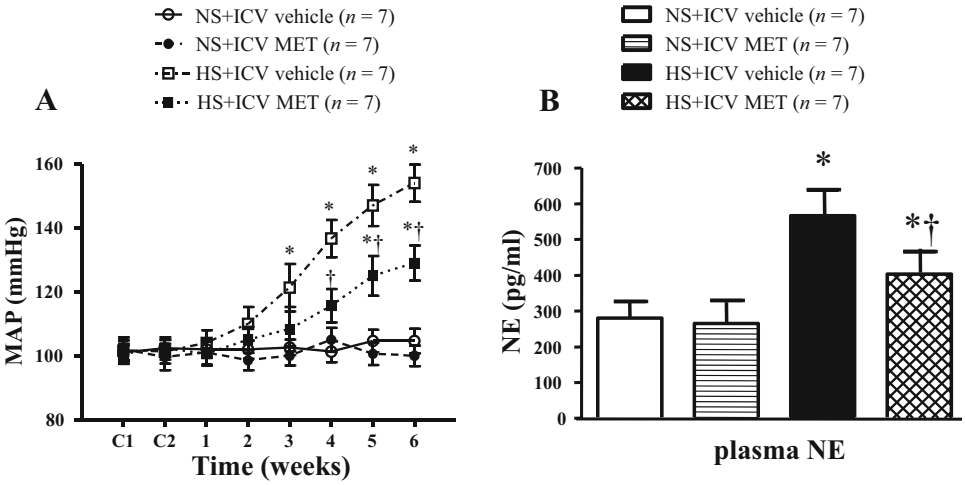

Fig. 1 Effects of ICV infusion of metformin (MET) on mean arterial pressure (MAP) and plasma norepinephrine (NE) levels in rats on a normal-salt (NS, 0.3% NaCl) or a high-salt (HS, 8% NaCl) diet. **A** MAP changes in the different groups. **B** Plasma NE levels in the different groups. Values are mean ± SEM. *$P < 0.05$ *versus* NS groups (NS + ICV vehicle or NS + ICV MET); †$P < 0.05$ HS + ICV MET *vs* HS + ICV vehicle.

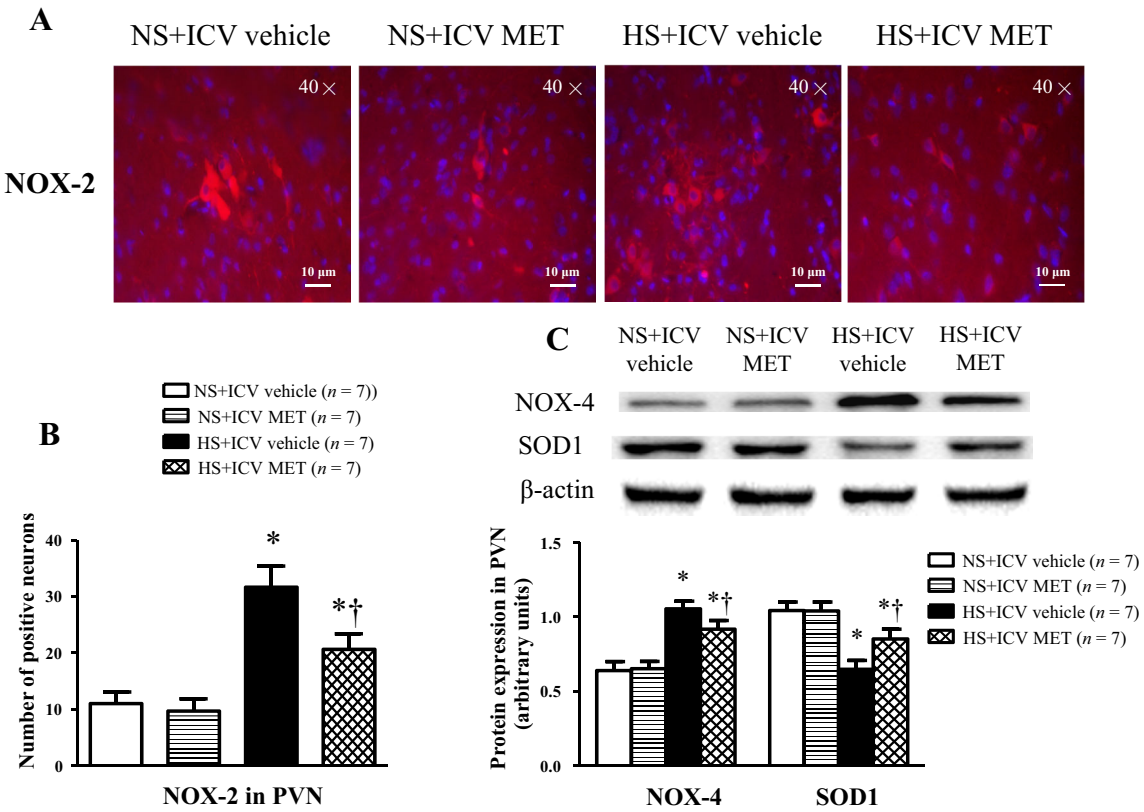

Fig. 2 Effects of ICV infusion of MET on NOX-2, NOX-4, and SOD expression in the PVN of NS and HS rats. **A** Immunofluorescence for NOX-2 (bright red) in the PVN in the different groups. Nuclei are labeled with DAPI and shown in blue. **B** Numbers of NOX-2-positive neurons in the PVN in the different groups. **C** A representative immunoblot and densitometric analysis of protein expression of NOX-4 and SOD in the PVN in the different groups. Values are mean ± SEM. $^{*}P < 0.05$ *vs* NS groups (NS + ICV vehicle or NS + ICV MET); $^{\dagger}P < 0.05$ HS + ICV MET *vs* HS + ICV vehicle.

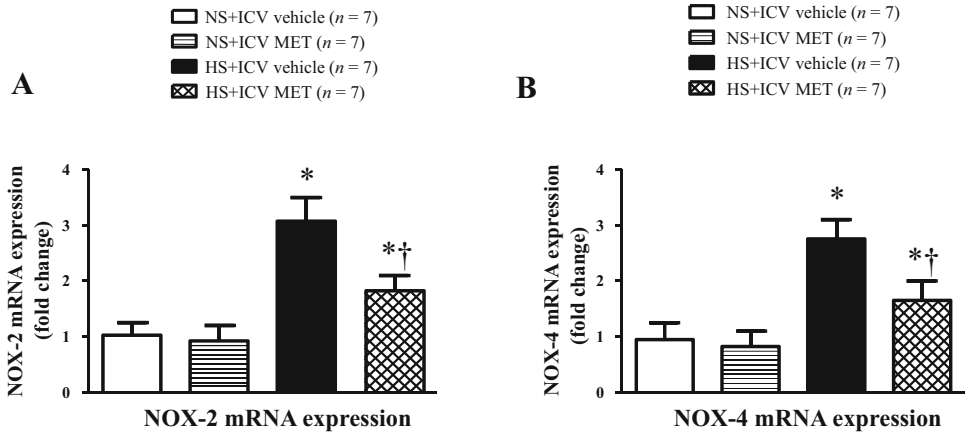

Fig. 3 Effects of ICV infusion of MET on mRNA expression of NOX-2 and NOX-4 in the PVN in NS and HS rats. **A** mRNA expression of NOX-2 in the PVN in the different groups. **B** mRNA expression of NOX-4 in the PVN in the different groups. $^{*}P < 0.05$ *vs* NS groups (NS + ICV vehicle or NS + ICV MET); $^{\dagger}P < 0.05$ HS + ICV MET *vs* HS + ICV vehicle.

(Fig. 2C) and activity (Fig. 4C) in the PVN in HS rats were reversed by MET treatment.

MET Reduces RAS Components in the PVN in Hypertensive Rats

The HS rats had higher PVN levels of ACE immunoreactivity (Fig. 5A, B) and AT1-R protein expression (Fig. 5C) than NS rats. This elevation in ACE (Fig. 5A, B) and AT1-R (Fig. 5C) expression was attenuated by ICV infusion of MET.

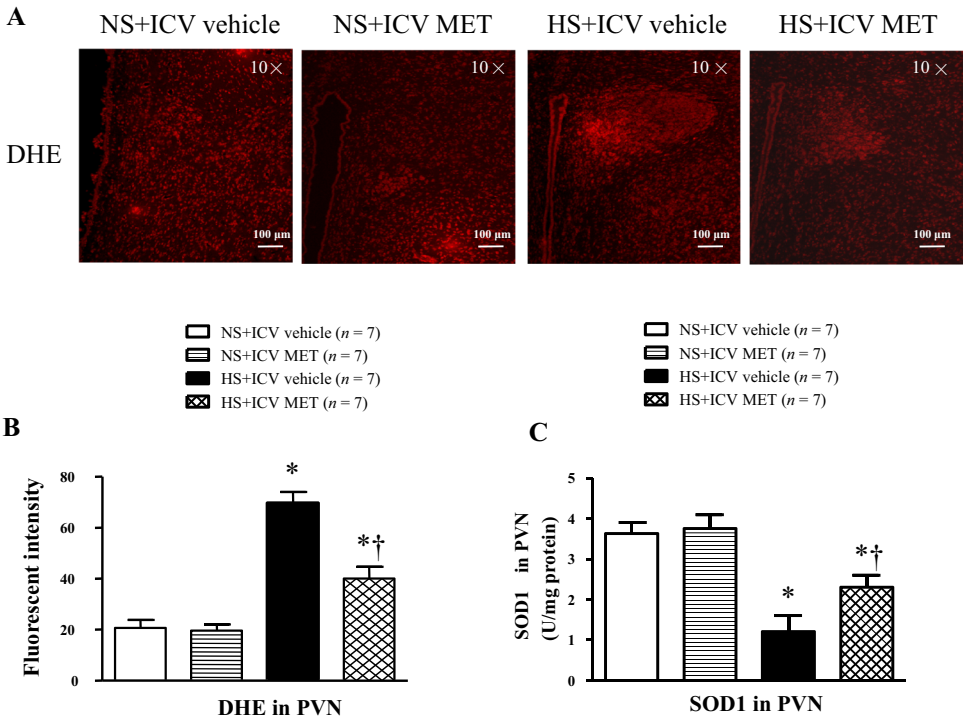

Fig. 4 Effects of ICV infusion of MET on ROS and SOD activity in the PVN in NS and HS rats. **A** Immunofluorescence images for superoxide (bright red) as determined by fluorescence-labeled dihydroethidium (DHE) in the PVN. **B** Superoxide in the PVN in the different groups. **C** SOD activity in the PVN in the different groups. $^{*}P < 0.05$ *vs* NS groups (NS + ICV vehicle or NS + ICV MET); $^{\dagger}P < 0.05$ HS + ICV MET *vs* HS + ICV vehicle.

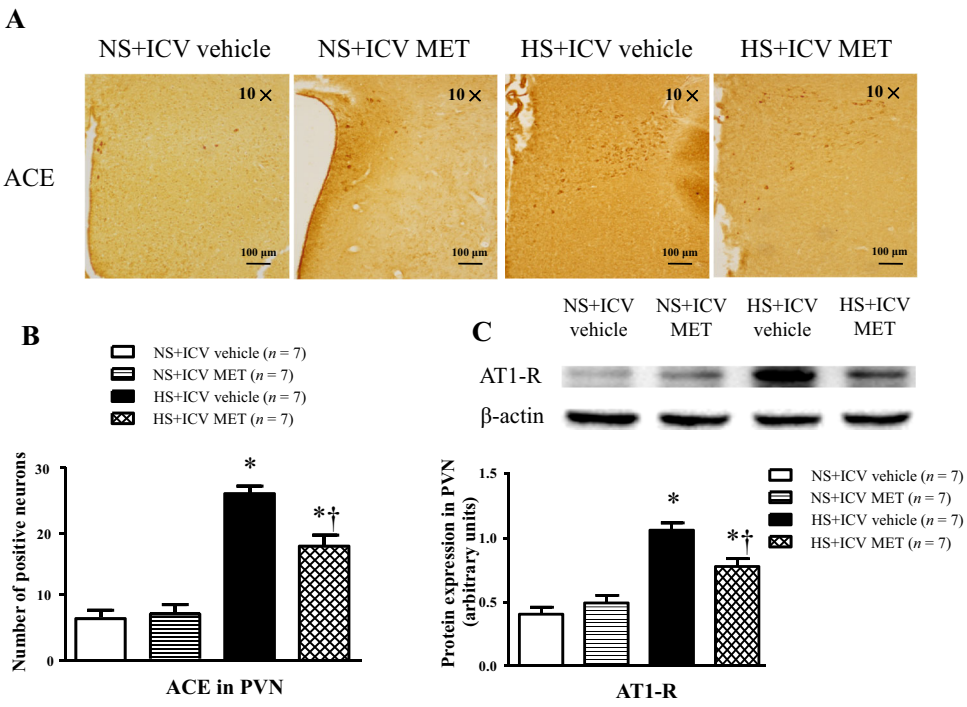

Fig. 5 Effects of ICV infusion of MET on expression of RAS components within the PVN in NS and HS rats. **A** Immunohistochemistry for ACE expression in the PVN in the different groups. **B** Numbers of ACE-positive neurons in the PVN in the different groups. C A representative immunoblot and densitometric analysis of protein expression of AT1-R in the PVN in the different groups. Values are mean ± SEM. $^{*}P < 0.05$ *vs* NS groups (NS + ICV vehicle or NS + ICV MET); $^{\dagger}P < 0.05$ HS + ICV MET *vs* HS + ICV vehicle.

MET Restores Neurotransmitters in the PVN in Hypertensive Rats

Higher PVN levels of NE (Fig. 6A) and glutamate (Fig. 6B) and a decreased level of GABA (Fig. 6C) were found in HS rats than in NS rats. MET treatment prevented the increase in NE (Fig. 6A) and glutamate (Fig. 6B), and the decrease in GABA (Fig. 2B) in the PVN in HS rats. Moreover, the PVN from HS rats showed a significant decrease in GAD67 immunoreactivity (Fig. 7A, B) and protein expression (Fig. 7C) as well as an increase in TH protein expression (Fig. 7C) compared with control rats.

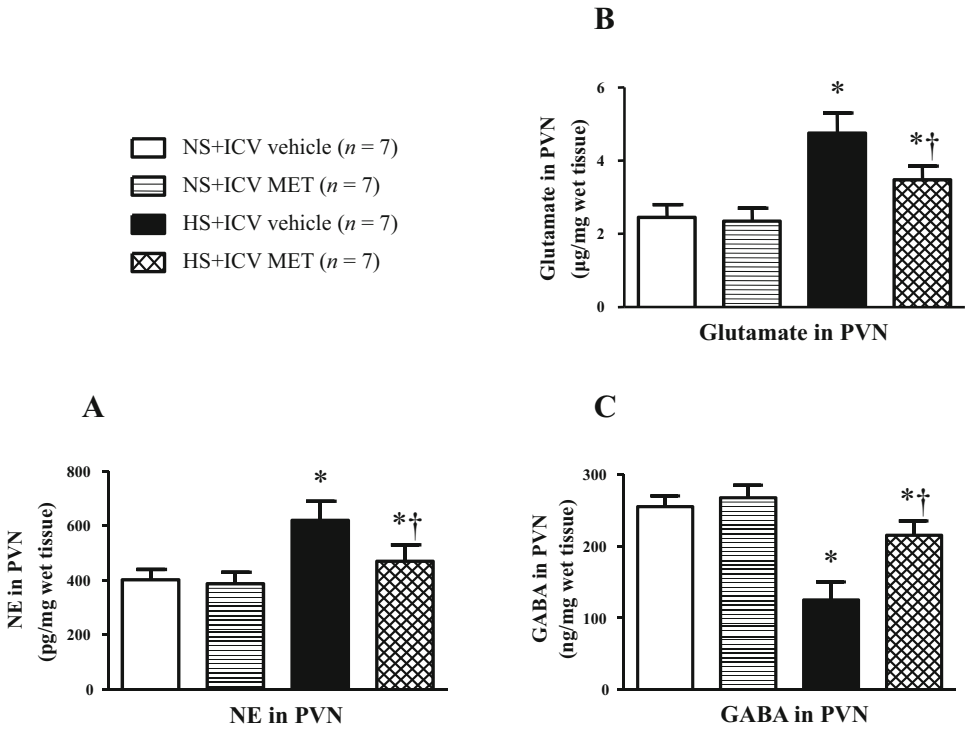

Fig. 6 Effects of ICV infusion of MET on the levels of norepinephrine (NE), glutamate and γ-aminobutyric acid (GABA) in the PVN in NS and HS rats. **A** NE levels in the PVN in the different groups. **B** Glutamate levels in the PVN in the different groups. **C** GABA levels in the PVN in the different groups. $^{*}P < 0.05$ *vs* NS groups (NS + ICV vehicle or NS + ICV MET); $^{\dagger}P < 0.05$ HS + ICV MET *vs* HS + ICV vehicle.

ICV infusion of MET increased GAD67 expression and decreased TH expression in HS rats (Fig. 7).

Discussion

Our results showed that an HS diet induced sympathoexcitation and hypertensive responses in salt-sensitive rats. Significant oxidative stress, RAS activation, and neurotransmitter imbalance were found in the PVN from these hypertensive rats. ICV infusion of MET notably attenuated blood pressure and sympathetic activity by suppressing oxidative stress, reducing RAS components, and restoring neurotransmitters in the PVN of hypertensive rats.

It is known that oxidative stress triggered by overproduction of ROS is one of the major mechanisms underlying the progression of hypertension [5, 33]. ROS in the PVN contribute to the regulation of sympathetic drive and blood pressure [32]. High salt results in excessive ROS, which contribute to hypertension *via* increasing sympathetic outflow [34]. In addition, high dietary salt raises cerebrospinal fluid Na^{+}, which can activate the RAS [35, 36]. ANG II activates NAD(P)H oxidase by interacting with AT1-R, leading to ROS production and sympathoexcitation [37, 38]. In our study, ROS production and the expression of NAD(P)H subunits (NOX-2 and NOX-4) together with MAP were markedly higher in HS rats than control NS rats. The RAS components (ACE and AT1-R) in the PVN were also higher. Our present work showed that ICV infusion of MET attenuated the above changes and increased the activity and expression of SOD in hypertensive rats. Moreover, NE, an indicator of sympathetic activity, showed markedly lower plasma levels in MET-treated hypertensive rats than in control rats. The reduction of RAS components and ROS production by MET has also been described in previous studies [39, 40]. These results suggest that the beneficial effect of central administration of MET in salt-sensitive hypertension is associated with restoring the balance between ROS and the antioxidant defense system.

Studies from our lab and others have indicated that ROS activation contributes to the imbalance of neurotransmitters [30, 41–43]. It is well established that the PVN is a vital cardiovascular regulatory center, and various neurotransmitters, such as NE, glutamate, and GABA are involved [44, 45]. Mounting evidence suggests that increased glutamatergic and adrenergic activity and decreased GABAergic activity in the PVN lead to sympathoexcitation and hypertensive responses [46–48]. Here, we found that HS rats had higher PVN levels of glutamate and NE, and a lower PVN level of GABA than NS rats. In addition, our results also found significantly higher TH expression and lower GAD67 expression (a marker for GABAergic neurons) in the PVN of HS rats than NS rats. Moreover, ICV infusion of MET prevented these increases in NE, glutamate, and TH and the reduction in GABA and GAD67 in the PVN of hypertensive rats.

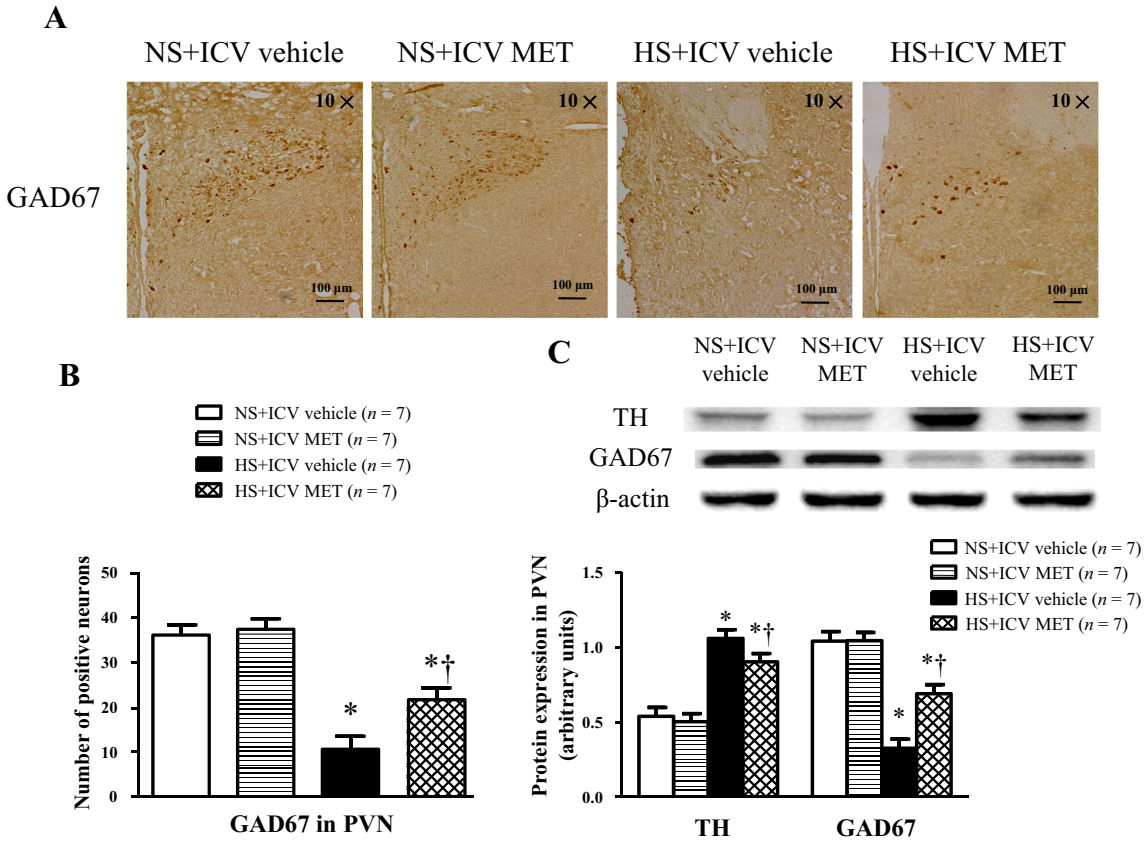

Fig. 7 Effects of ICV infusion of MET on the expression of tyrosine hydroxylase (TH) and the 67-kDa isoform of glutamate decarboxylase (GAD67) in the PVN in NS and HS rats. **A** Immunohistochemistry for GAD67 expression in the PVN. **B** Numbers of GAD67-positive neurons in the PVN in the different groups. **C** A representative immunoblot and densitometric analysis of protein expression of TH and GAD67 in the PVN in the different groups. $^{*}P < 0.05$ *vs* NS groups (NS + ICV vehicle or NS + ICV MET); $^{\dagger}P < 0.05$ HS + ICV MET *vs* HS + ICV vehicle.

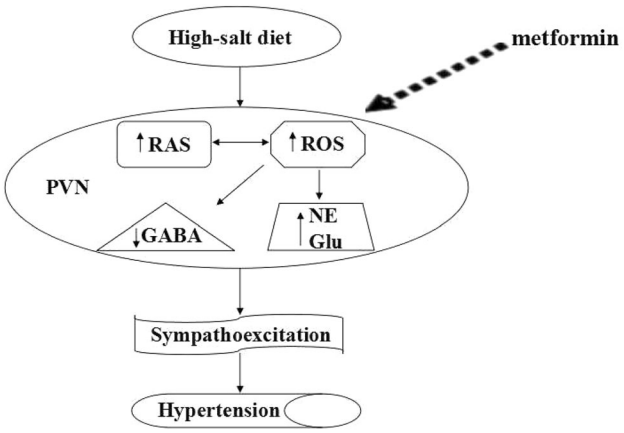

Fig. 8 Schematic showing the proposed pathways of the effects of ROS on regulating sympathetic activity and the blood pressure response within the PVN in high salt-sensitive hypertension.

In addition, Staruschenko and colleagues investigated the effects of continuous venous infusion (6.9 μL/min) of MET (200 mg/kg per day for 3 weeks) on salt-induced hypertension in Dahl salt-sensitive rats [49]. The MET treatment in the rats with high-Na^{+} treatment had no effect on the pattern of hemodynamic changes: neither MAP, circadian rhythm, nor HR differed between the vehicle and MET-treated groups. They concluded that MET treatment did not activate 5′-AMP-activated protein kinase and its downstream pathways, which is associated with the regulation of epithelial Na^{+} channel-dependent short-circuit currents, and did not find any effect of MET on salt-induced hypertension in these rats. However, we treated Dahl salt-sensitive rats with salt-induced hypertension using ICV infusion of MET (25 μg/day) for 6 weeks. Compared with the Staruschenko study, the ICV infusion of MET had a sustained sympathoinhibitory effect in the central nervous system, consistent with the study of Petersen *et al*. [24]. So, our study provides evidence to support the conclusion that central MET attenuates sympathetic activity and blood pressure by restoring the balance between excitatory and inhibitory neurotransmitters in the PVN in salt-sensitive hypertensive rats (Fig. 8).

Acknowledgements We gratefully acknowledge Jian-Jun Mu (Department of Cardiology, The First Affiliated Hospital of Xi'an

Jiaotong University) for providing the Dahl salt-sensitive rats. This work was supported by the National Natural Science Foundation of China (81600333, 81770426, 81800372, 91439120, and 91639105), the Postdoctoral Science Foundation of China (2016M602835, 2017M620457), and the Postdoctoral Science Foundation of Shaanxi Province, China (2016BSHEDZZ91).

Conflict of interest The authors declare no competing financial interests.

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