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ORIGINAL ARTICLE

Chronic Intermittent Hypobaric Hypoxia Ameliorates Renal Vascular Hypertension Through Up-regulating NOS in Nucleus Tractus Solitarii

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Abstract Chronic intermittent hypobaric hypoxia (CIHH) is known to have an anti-hypertensive effect, which might be related to modulation of the baroreflex in rats with renal vascular hypertension (RVH). In this study, RVH was induced by the 2-kidney-1-clip method (2K1C) in adult male Sprague-Dawley rats. The rats were then treated with hypobaric hypoxia simulating 5000 m altitude for 6 h/day for 28 days. The arterial blood pressure (ABP), heart rate (HR), and renal sympathetic nerve activity (RSNA) were measured before and after microinjection of *L*-arginine into the nucleus tractus solitarii (NTS) in anesthetized rats. Evoked excitatory postsynaptic currents (eEPSCs) and spontaneous EPSCs (sEPSCs) were recorded in antero-gradely-labeled NTS neurons receiving baroreceptor afferents. We measured the protein expression of neuronal nitric oxide synthase (nNOS) and endothelial NOS (eNOS) in the NTS. The results showed that the ABP in RVH rats was significantly lower after CIHH treatment. The inhibition of ABP, HR, and RSNA induced by *L*-arginine was less in RVH rats than in sham rats, and greater in the CIHH-treated RVH rats than the untreated RVH rats. The eEPSC amplitude in NTS neurons receiving baroreceptor afferents was lower in the RVH rats than in the sham rats and recovered after CIHH. The protein expression of nNOS and eNOS in the NTS was lower in the RVH rats than in the sham rats and this decrease was reversed by CIHH. In short, CIHH treatment decreases ABP in RVH rats *via* up-regulating NOS expression in the NTS.

Keywords Chronic intermittent hypobaric hypoxia · Renal vascular hypertension · Nitric oxide · Renal sympathetic nerve activity · Excitatory postsynaptic current

Introduction

Hypertension is a common cardiovascular disease and a major risk factor for stroke, coronary heart disease, and other cardiovascular diseases. Although many anti-hypertensive medications have been developed for the treatment of various types of hypertension [1], its prevention and treatment still face challenges because some hypertensive patients are unresponsive to the available anti-hypertensive drugs [2]. Furthermore, the clinical use of these anti-hypertensive medications is limited by their side-effects [3]. Therefore, it is important to seek effective therapeutics for hypertension. It has been reported that chronic intermittent hypobaric hypoxia (CIHH) decreases arterial blood pressure (ABP) in patients with essential hypertension and in spontaneously hypertensive rats [4, 5]. Our previous study demonstrated that CIHH decreases ABP in rats with renovascular hypertension (RVH) [6].

The baroreflex plays an important role in the homeostasis of blood pressure under physiological conditions, and hypertension is closely associated with the resetting and/or impairment of baroreflex function. Numerous studies have

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shown that the baroreflex is impaired in a variety of animal models of hypertension (such as spontaneously hypertensive rats, RVH, and metabolic syndrome hypertension) and in human hypertension [5]. Our previous study has shown that CIHH facilitates baroreflex function, a mechanism of the anti-hypertensive effect of CIHH in RVH rats [6, 7]. However, the molecular and cellular mechanisms underlying the CIHH-induced facilitation of the baroreflex have not been elucidated.

The nucleus tractus solitarii (NTS) is one of the critical central nuclei receiving visceral sensory inputs including baroreceptor afferents. The aortic depressor nerve afferents release glutamate onto second-order NTS neurons [8]. Nitric oxide (NO) regulates the activity of NTS neurons by affecting synaptic inputs under physiological as well as pathophysiological conditions [9].

NO is synthesized from its precursor *L*-arginine by neuronal (nNOS) and endothelial (eNOS) NO synthases in the central nervous system, including in the NTS [10]. NO not only induces vasorelaxation directly, but also regulates cardiovascular function *via* the central nervous system. It has been demonstrated that CIHH increases the expression levels of inducible NOS mRNA and protein in the left ventricle of rats, a mechanism involved in the cardioprotective effect of CIHH [11]. However, it is not clear whether NO production in the NTS is involved in the CIHH-induced facilitation of the baroreflex and in the anti-hypertensive effect. Thus, we designed this study to determine the effect of CIHH on second-order NTS neurons receiving baroreceptor afferents in RVH rats and the underlying neuronal mechanisms.

Materials and Methods

Animal Grouping and CIHH Treatment

Adult male Sprague-Dawley rats weighing 170 g–190 g were used in this study. All the experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The experimental protocols were approved by the Committee on the Use of Animals for Teaching and Research of Hebei Medical University.

The animals were provided by the Experimental Animal Center of Hebei Province and were randomly divided into four groups: sham-operated group (SHAM), RVH group (RVH), CIHH treatment plus sham-operated group (CIHH + SHAM), and RVH plus CIHH treatment group (RVH + CIHH). The hypertension was induced by the two-kidney-1-clip (2K1C) method. Rats in the CIHH + SHAM group were treated with hypobaric hypoxia simulating 5000 m altitude (P_B = 404 mmHg, Po_2 = 84 mmHg) in a hypobaric chamber for 28 days, 6 h/day, as well as a sham abdominal operation [12]. RVH + CIHH rats were subjected to both CIHH and 2K1C. SHAM rats received abdominal surgery without arterial clamping and CIHH.

The systolic blood pressure (BP) in conscious rats was measured by tail-cuff plethysmography (LE5001, Panlab, Barcelona, Spain) at fixed time each week. One hour prior to measuring BP, the rats were put into a chamber isolated from noise and at ~30 °C to induce dilation of the tail artery. After three consecutive days of adaption, the BP and heart rate (HR) were measured. The BP was measured three times and the average was recorded.

Rat Model of Renovascular Hypertension

RVH was induced using the Goldblatt 2K1C method as described previously [13]. Briefly, the rats were anesthetized with sodium pentobarbital [35 mg/kg, intraperitoneal (i.p.) injection] and a retroperitoneal flank incision was made. The right renal artery was exposed and partially occluded by a U-shaped silver clip with an internal diameter of 0.20 mm. The operation was performed under strict sterile condition and antibiotics were used for three days after surgery. The rats were kept in their home cages for 4 weeks to allow full recovery from the operation. SHAM rats were subjected to a similar surgical procedure but without clip placement. The criterion for success was a systolic BP >150 mmHg, and only 2K1C rats satisfying this criterion were used in experiments [14].

Recording Hemodynamics and Sympathetic Nerve Activity *In Vivo*

The rats were anesthetized with 25% urethane and 10% chloral hydrate (2:1, 0.5 mL/100 g, i.p.) and additional anesthetics were used as needed to maintain sufficient anesthesia, which was indicated by stable ABP and HR. The body temperature was maintained at 37 ± 0.5 °C using a heating pad throughout experiments. The trachea was cannulated and the rats were ventilated mechanically with 100% O_2 (Harvard Apparatus Inc., Holliston, MA). The left femoral artery was cannulated for ABP measurement and the right femoral vein was used for drug administration.

The left kidney was exposed *via* a retroperitoneal approach. A branch of the renal sympathetic nerve was carefully isolated from the surrounding tissue and clamped distally to eliminate afferent activity. The isolated nerve was placed on a bipolar silver electrode for nerve discharge recording and immediately immersed in warm liquid paraffin (37 °C) to avoid nerve drying and interference. Renal sympathetic nerve activity (RSNA) was filtered (160 Hz–1000 Hz) and amplified with a biological

experiment system (PowerLab, ADInstruments, Bella Vista, Australia; DP301 amplifier, Warner Instruments, Hamden, CT). The amplified signal was integrated (160 ms) and acquired with software. At the end of each experiment, the proximal end of the nerve was clamped to block the discharge and the noise level was determined as the difference after clamping. The RSNA was obtained by subtraction of the noise level from total RSNA and expressed as percentage of baseline, which was the nerve activity before any experimental manipulation. We integrated the nerve activity (with subtraction of background noise), set the basal level at 100%, and the percentage change from the baseline value was calculated.

Microinjection

The head of the rat was fixed on a stereotaxic frame inclined 45 °C downward for better visualization of the calamus scriptorius, and an occipital craniotomy was performed to expose the dorsal surface of the medulla in the region of the obex. *L*-arginine (10 nmol, 50 nL) was ejected bilaterally through a glass pipette over 2 s by application of pressurized air to the pipette using a microinjection system (KDS100, KD Scientific, Holliston, MA). The tip of the injection pipette was inserted into the medial part of NTS according to the coordinates: 0.4 mm–0.5 mm rostral, 0.5 mm–0.6 mm lateral to the calamus scriptorius, and 0.4 mm below the dorsal surface of the medulla. The injection was monitored under an operating microscope. The injection volume was determined by viewing the movement of the fluid meniscus inside the pipette by a microscope with a calibrated micrometer. Accurate pipette location and the spread of injection were verified histologically. At the end of the experiment, 50 nL of 2% Chicago blue dye was ejected from the same pipette at the same site. After euthanasia, the brainstem was removed rapidly and slices containing NTS were prepared and visualized under a biological microscope. The dye spot and spread area were identified and plotted on standardized sections according to the atlas of Paxinos and Watson [15] (Fig. 1). Data were excluded if the microinjection site and spread were beyond the area of the NTS.

Western Blot Analysis

Rats were deeply anesthetized with 25% urethane (i.p.) and decapitated. The brain was harvested and immersed in ice-cold artificial cerebrospinal fluid (aCSF). Coronal slices (300-μm thick) were cut at the brainstem level using vibrating microtome (1200S, Leica, Wetzlar, Germany). The NTS region was punched out from the slices using a blunt 19-gauge needle. The NTS tissue was pooled from 6–8 rats and frozen at −80 °C until blotting. The

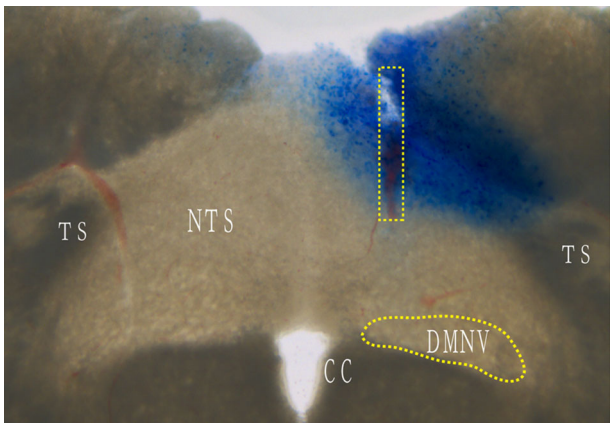

Fig. 1 Photomicrograph showing the location of microinjection in the NTS. The brainstem slices containing the NTS were prepared shortly after 50 nL of 2% Chicago blue dye was injected. The injection track and area of dye spread was visualized under a microscope. The dashed line indicates the injection track. CC, central canal; DMNV, dorsal motor nucleus of vagus; TS, solitary tract.

experiment was repeated in three separate groups of rats and tissues. The NTS was homogenized in lysis buffer and then centrifuged at 12,000 rpm for 15 min at 4 °C. Protein concentration was determined using the Brayford assay (Multi Sciences Biotech Co, Hangzhou, China). The proteins nNOS, eNOS, and GAPDH were separated on an Immobilon-P Transfer membrane (Millipore Corp., Bedford, MA) and the proteins on the membrane were immunoblotted by antibodies against nNOS (rabbit anti-rat 1:800; Abgent, San Diego, CA) and eNOS (rabbit anti-rat 1:500; Abcam, Hong Kong). The same membrane was stripped and re-blotted with an anti-GAPDH antibody (rabbit anti-rat 1:5000; GeneTex, Irvine, CA) for normalization. Blots were developed by the chemiluminescent detection method (Amersham ECL, Chicago, IL). The protein blots were quantified by densitometry using ImageJ software (NIH, USA) and normalized to GAPDH.

In Vitro Electrophysiological Experiments

NTS neurons receiving baroreceptor afferents were labeled anterogradely with 4-(4-dihexadecylamino) styryl-N-methylpyridinium iodide (Dil), as described previously [16]. Briefly, the aortic depressor nerve (ADN) was carefully exposed in anesthetized rats. The Dil was put on the ADN at the point where it enters the nodose ganglion. After operation, the rats were treated prophylactically with an antibiotic (ceftriaxone sodium, 100 mg/kg, i.p., daily for 3 days) and an analgesic (buprenorphine, 0.5 mg/kg, subcutaneous injection, every 12 h for 2 days). The rats were then returned to their home cages for 2–3 weeks to allow the Dil to be transported to the NTS.

Slices containing the NTS were prepared from the Dil-treated rats, as described previously [17]. Briefly, the rats were decapitated under anesthesia with 25% urethane, and the brain was quickly removed and placed in ice-cold aCSF containing the following (in mmol/L): 124.0 NaCl, 3.0 KCl, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 1.4 NaH_2PO_4, 10.0 glucose, and 26.0 $NaHCO_3$, which was saturated with a mixture of 95% O_2 and 5% CO_2. A tissue block containing the NTS was trimmed and glued onto the stage of a vibrating microtome (1200S, Leica). Coronal slices (250 μm–300 μm thick) were cut and pre-incubated in aCSF continuously gassed with 95% O_2 and 5% CO_2 at 34 °C for 1 h before recording.

The slices were placed in a recording chamber continuously perfused with aCSF (saturated with 95% O_2 and 5% CO_2) at 3.0 mL/min at 34 °C maintained by an inline solution heater. The labeled NTS neurons were first identified under an upright microscope equipped with epifluorescence illumination and differential interference contrast optics. The evoked (eEPSCs) and spontaneous (sEPSCs) excitatory postsynaptic currents in NTS neurons receiving baroreflex afferents were recorded *via* electrodes pulled from borosilicate capillaries on a micropipette P-97 puller (Sutter Instruments, Novato, CA). The electrode resistance was 3 MΩ–6 MΩ when filled with a solution containing (mmol/L) 131 potassium gluconate, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 EGTA, 4 ATP-Mg, adjusted to pH 7.25 with 1 mol/L KOH, 270 mOsm–290 mOsm. After a tight gigaohm seal was formed between microelectrode and cell membrane, a negative pressure was used to rupture the cell membrane and establish the whole-cell mode. In the whole-cell voltage clamp mode, the postsynaptic currents in NTS neurons receiving baroreflex afferents were recorded. At a holding potential of −70 mV, the ipsilateral tractus solitarius (TS) was stimulated (10 V and 0.1 ms square wave) to trigger synaptic currents by a biopolar tungsten electrode connected to a stimulator (tip 120 μm, impedance 10 mΩ). The distance between the stimulating electrode and the recorded neuron was 200 μm–500 μm. Single synaptic eEPSCs evoked by 20 Hz electrical stimulation were confirmed based on the characteristics of short latency. In the presence of bicuculline (10 μmol/L, a $GABA_A$ receptor antagonist) or *L*-arginine (100 μmol/L, a precursor of NO), eEPSCs and sEPSCs were evoked and recorded. eEPSCs and sEPSCs were processed using a Multiclamp 700B amplifier and Digidata 1440A (Axon Instruments, Foster, CA). The recording was abandoned if the input resistance changed >15% during the recording. In all cases, values were obtained and averaged during a 3-min to 5-min recording period before and after drug application.

Drugs

L-Arginine was from Sigma (St. Louis, MO). Bicuculline was purchased from Tocris. Drugs were dissolved in aCSF and prepared before experiments.

Data Analysis

For the *in vivo* data, BP and HR were determined from the arterial pressure pulse (PowerLab, ADInstruments). RSNA signals were rectified at a time constant of 1 s and integrated off-line using the DP301 amplifier. The nerve activity was obtained by subtracting background noise. Control values of nerve activity were obtained by averaging the signal over a 60-s period immediately before each treatment. Response values following each intervention were averaged over 30 s when the maximal responses occurred. The eEPSCs and sEPSCs were analyzed using Clampfit (Molecular Device, San Jose, CA). To compare the BP, RSNA, and HR responses to the agent microinjected within a group, we performed a repeated-measures analysis of variance (ANOVA) with Dunnett's *post-hoc* test. A two-way ANOVA with Bonferroni's *post-hoc* test was used to compare responses in four groups. $P < 0.05$ was considered statistically significant.

Results

CIHH Decreased Arterial Blood Pressure in RVH Rats

The systolic BP was significantly higher in RVH rats than in SHAM rats three weeks after 2K1C operation (161.2 ± 6.5 *vs* 104.8 ± 4.5 mmHg, $n = 6$ rats/group, $P < 0.01$). CIHH treatment significantly reduced the systolic BP in RVH rats (158.4 ± 7.5 *vs* 136.2 ± 3.7 mmHg, $P < 0.01$) but had no effect on systolic BP in SHAM rats (104.8 ± 4.5 *vs* 106.0 ± 5.1 mmHg, $P > 0.05$) (Fig. 2). These data indicated that CIHH treatment has an anti-hypertensive effect in RVH rats.

CIHH Enhanced Sympathetic Inhibition Induced by Microinjection of *L*-Arginine into the NTS of RVH Rats

The baseline of mean BP, RSNA, and HR are shown in Table 1. Microinjection of the NO precursor *L*-arginine (10 nmol, 50 nL) decreased RSNA, mean ABP (MAP) and HR in all groups (Fig. 3). Compared with SHAM rats (ΔRSNA, 10.3% ± 0.5%; ΔMAP, 15.0% ± 1.9%; ΔHR, 10.4% ± 2.4%), the *L*-arginine-induced decreases in

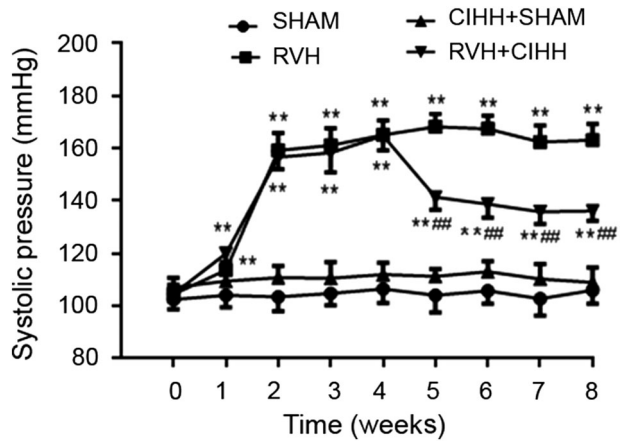

Fig. 2 Effect of CIHH on systolic blood pressure in renal vascular hypertensive rats. SHAM, sham group; RVH, renal vascular hypertension group; CIHH + SHAM, chronic intermittent hypobaric hypoxia group; RVH + CIHH, RVH group receiving CIHH treatment. All data are expressed as mean ± SEM, $n = 6$/group. $^{**}P < 0.01$ compared with SHAM group, $^{\#\#}P < 0.01$ compared with RVH group.

RSNA, MAP and HR were significantly attenuated in RVH rats (ΔRSNA, 4.6% ± 0.2%; ΔMAP, 6.4% ± 1.5%; ΔHR, 4.5% ± 0.6%; $P < 0.05$–0.01, $n = 8$ rats/group). The decreases in RSNA, MAP, and HR were greater in CIHH + SHAM rats (ΔRSNA, 11.3% ± 0.4%; ΔMAP, 20.9% ± 4.5%; ΔHR, 13.2% ± 2.8%) and RVH + CIHH rats (ΔRSNA, 9.6% ± 0.5%, ΔMAP, 17.0% ± 1.3%; ΔHR, 10.9% ± 2.4%) than in RVH rats ($P < 0.01$, $n = 8$ rats/group, Fig. 3).

These results suggested that the *L*-arginine-induced sympathetic inhibition is decreased in RVH rats and CIHH treatment restores the decreased sympathetic inhibition in these rats.

CIHH Facilitated eEPSCs and sEPSCs in Second-Order NTS Neurons

The labeled baroreceptor NTS neurons in brainstem slices were identified under an upright microscope with a combination of epifluorescence illumination and infrared light (Fig. 4). The eEPSCs were recorded at a holding potential of −70 mV in the presence of the $GABA_A$ receptor antagonist bicuculline (10 μmol/L). Compared with the SHAM group (111.0 ± 11.7 pA, $n = 20$ NTS neurons), the average amplitude of eEPSCs in NTS neurons receiving baroreceptor afferents was decreased in the RVH group (77.2 ± 11.7 pA, $n = 23$ NTS neurons $P < 0.01$). Compared with the RVH group, the amplitude of eEPSCs was increased significantly in the CIHH + SHAM group (124.6 ± 4.8 pA, $n = 13$ NTS neurons) and the RVH + CIHH group (95.1 ± 6.2 pA, $n = 15$ NTS neurons, $P < 0.01$, Fig. 5).

Compared with SHAM rats (8.9 ± 1.0 Hz, $n = 14$ NTS neurons), the frequency of sEPSCs was significantly higher in RVH rats (13.2 ± 0.9 Hz, $n = 10$ NTS neurons, $P < 0.05$). The sEPSC frequency of baroreceptor-sensitive NTS neurons in CIHH + SHAM rats (20.7 ± 2.8 Hz, $n = 12$ NTS neurons) and RVH + CIHH rats (17.2 ± 1.1 Hz, $n = 12$ NTS neurons) was significantly higher than that in RVH rats ($P < 0.05$, Fig. 6). There were no significant differences in the amplitudes of sEPSCs in baroreceptor-sensitive NTS neurons among SHAM (14.1 ± 1.7 pA), RVH (18.1 ± 4.4 pA), CIHH + SHAM (13.0 ± 1.6 pA), and RVH + CIHH rats (19.3 ± 2.1 pA, $P > 0.05$; Fig. 6).

These results indicated that CIHH treatment facilitates excitatory synaptic inputs to NTS neurons receiving baroreceptor afferents.

CIHH Enhanced the *L*-Arginine-Induced Increases in eEPSCs and sEPSCs in Baroreceptor-Sensitive NTS Neurons

Since synaptic glutamate release is involved in the NO-induced increase in the firing activity of autonomic NTS neurons, we determined the effect of *L*-arginine on glutamatergic synaptic inputs to the baroreceptor-sensitive NTS neurons. The eEPSCs were recorded in the presence of 1 μmol/L gabazine and 5 μmol/L strychnine at a holding potential of −70 mV. The eEPSCs were elicited by electrical stimulation of the TS ipsilateral to the recording site. *L*-arginine (100 μmol/L) increased the peak amplitude

Table 1 The baseline MAP, RSNA and HR in anesthetized rats.

	MAP (mmHg)	RSNA (mV.s)	Heart rate (bpm)
SHAM	79.1 ± 11.40	0.22 ± 0.06	351.0 ± 55.19
RVH	119.0 ± 10.73**	0.46 ± 0.10**	370.0 ± 18.83
CIHH + SHAM	67.7 ± 8.98	0.18 ± 0.03	333.4 ± 38.14
RVH + CIHH	72.0 ± 8.05$^{\#\#}$	0.29 ± 0.07$^{\#\#}$	318.2 ± 39.33$^{\#}$

MAP: mean arterial pressure, RSNA: renal sympathetic nerve activity, HR: heart rate; SHAM: Sham group, RVH: renal vascular hypertension group, CIHH + SHAM: chronic intermittent hypobaric hypoxia group, RVH + CIHH: RVH plus CIHH group. All data are expressed as mean ± SEM; $n = 6$ for each group. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* SHAM; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* RVH.

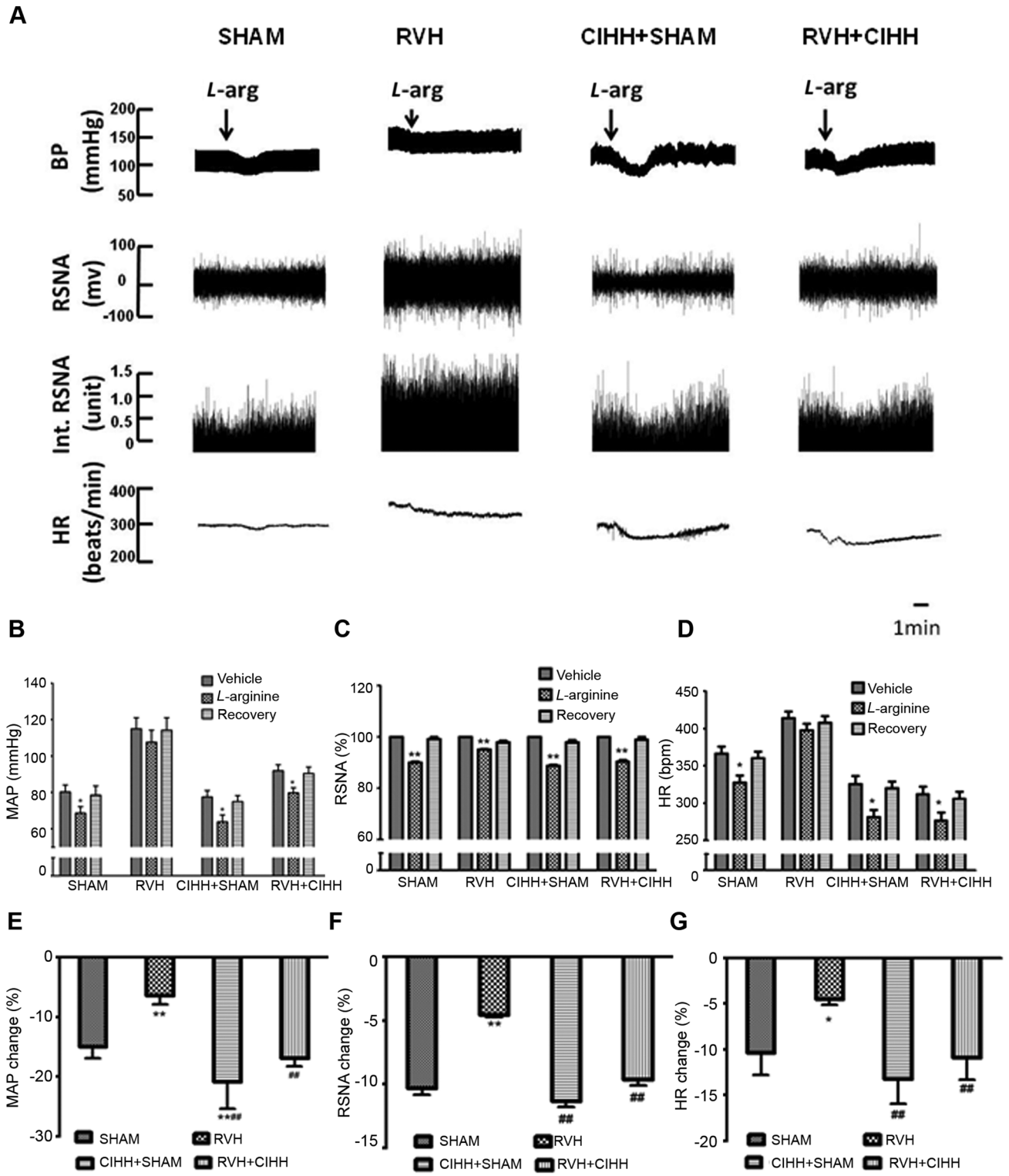

Fig. 3 Effect of microinjection of *L*-arginine into the NTS on mean arterial pressure (MAP), renal sympathetic nerve activity (RSNA), and heart rate (HR) in rats. **A** Representative traces of MAP, RSNA, and HR. **B–D** Summary data showing that the *L*-arginine-induced sympathoinhibitory response was decreased in the RVH group. Note that CIHH treatment decreased MAP, RSNA and HR in RVH rats and restored the *L*-arginine-induced decreased sympathoinhibitory response in these rats. **E–G** Summary data showing changes in MAP, RSNA, and HR after *L*-arginine microinjection. SHAM, sham group; RVH, renal vascular hypertension group; CIHH + SHAM, chronic intermittent hypobaric hypoxia group; RVH + CIHH, RVH group receiving CIHH treatment. All data are expressed as mean ± SEM, $n = 6$/group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with SHAM group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with RVH group.

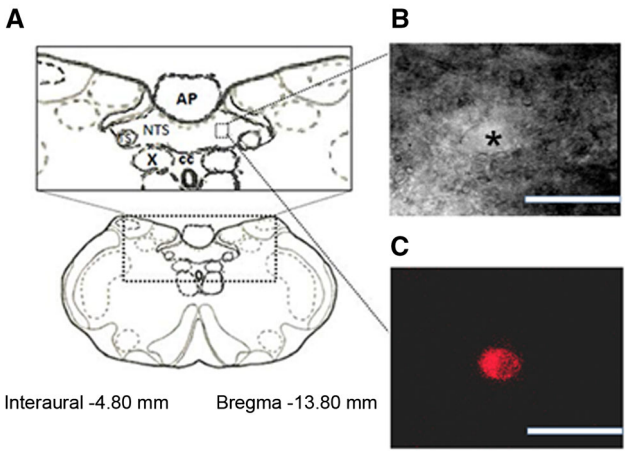

Fig. 4 Anterogradely-labeled NTS neuron in a brain slice. **A** Diagram showing the location of the NTS at the bregma −13.80 mm level. **B**, **C** Photomicrographs showing an NTS neuron in contact with DiI-labeled baroreceptor afferent terminals viewed under differential interference contrast optics (**B**, indicated by *) and under epifluorescence illumination (**C**, red). Scale bars: 20 μm.

of eEPSCs in baroreceptor-sensitive NTS neurons of SHAM rats (from 97.5 ± 5.6 pA to 124.3 ± 9.6 pA, Δ 27.1% ± 2.4%, n = 21 NTS neurons, $P < 0.05$), RVH rats (from 71.1 ± 4.8 pA to 80.8 ± 6.0 pA, Δ 11.6% ± 2.1%, n = 29 NTS neurons, $P < 0.05$), CIHH + SHAM rats (from 115.7 ± 6.3 pA to 159.1 ± 3.4 pA, Δ 39.2% ± 3.5%, n = 15 NTS neurons, $P < 0.05$), and RVH + CIHH rats (from 95.1 ± 5.9 pA to 114.8 ± 5.2 pA, Δ 22.4% ± 3.4%, n = 19 NTS neurons, $P < 0.05$) (Fig. 7). The increase of *L*-arginine-induced eEPSCs in baroreceptor-sensitive NTS neurons was less in RVH rats than that in SHAM rats. And the increase of eEPSCs induced by *L*-arginine in CIHH + SHAM and RVH + CIHH rats was higher than that in RVH rats ($P < 0.05$, Fig. 7).

Similarly, experiments were performed on anatomically identified baroreceptor-sensitive NTS neurons to determine the effect of NO on the firing activity of these neurons in the four groups of rats. The NO donor *L*-arginine (100 μmol/L) increased the sEPSCs frequency in baroreceptor-sensitive NTS neurons in SHAM (from 7.8 ± 1.3 Hz to 11.3 ± 2.0 Hz, Δ 46.2% ± 9.1%, n = 21 NTS neurons, $P < 0.05$), RVH (from 16.3 ± 2.6 Hz to 19.6 ± 2.0 Hz, Δ 19.2% ± 3.0%, n = 19 NTS neurons, $P > 0.05$), CIHH + SHAM (from 19.0 ± 1.5 Hz to 31.9 ± 1.6 Hz, Δ 61.6% ± 16.9%, n = 16 NTS neurons, $P < 0.05$), and RVH + CIHH rats (from 17.1 ± 1.7 Hz to 23.6 ± 0.8 Hz, Δ 40.3% ± 4.5%, n = 22 NTS neurons, $P > 0.05$). The increase of sEPSCs frequency induced by 100 μmol/L *L*-arginine in baroreceptor-sensitive NTS neurons was less in RVH rats than in SHAM, CIHH + SHAM, and RVH + CIHH rats ($P < 0.01$, Fig. 8). There were no significant differences in the sEPSC amplitude in baroreceptor-sensitive NTS neurons in each group before *versus* after *L*-arginine application (Fig. 8).

These results indicated that CIHH treatment significantly facilitates NO-induced enhancement of excitatory synaptic inputs to NTS neurons receiving baroreceptor afferents.

CIHH Increased nNOS and eNOS Expression in the NTS of RVH Rats

The protein expression levels of nNOS and eNOS in the NTS were lower in RVH rats than in SHAM rats (Fig. 9). And these levels were significantly higher in SHAM +

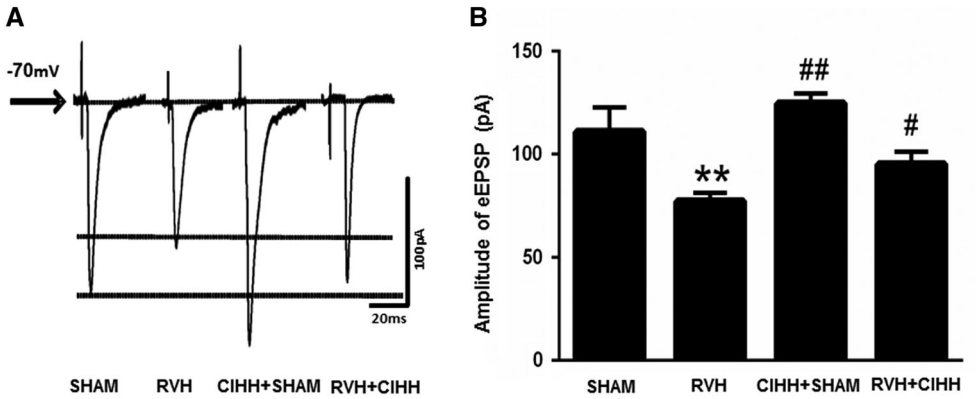

Fig. 5 Effect of CIHH on evoked excitatory postsynaptic currents (eEPSCs) in labeled NTS neurons in rats. **A**, **B** Representative raw traces of eEPSCs recorded from NTS neurons (**A**) and summary data (**B**) showing that the amplitude of eEPSCs was decreased in the NTS neurons of RVH rats, while CIHH treatment restored the decreased eEPSCs. SHAM, sham group, n = 20 NTS neurons; RVH, renal vascular hypertension group, n = 23 NTS neurons; CIHH + SHAM, chronic intermittent hypobaric hypoxia group, n = 13 NTS neurons; RVH + CIHH, RVH group receiving CIHH treatment, n = 15 NTS neurons. Traces in **A** are averages of 10 consecutive sweeps in each neuron. Data are expressed as mean ± SEM, $^{**}P < 0.01$ compared with SHAM group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with RVH group.

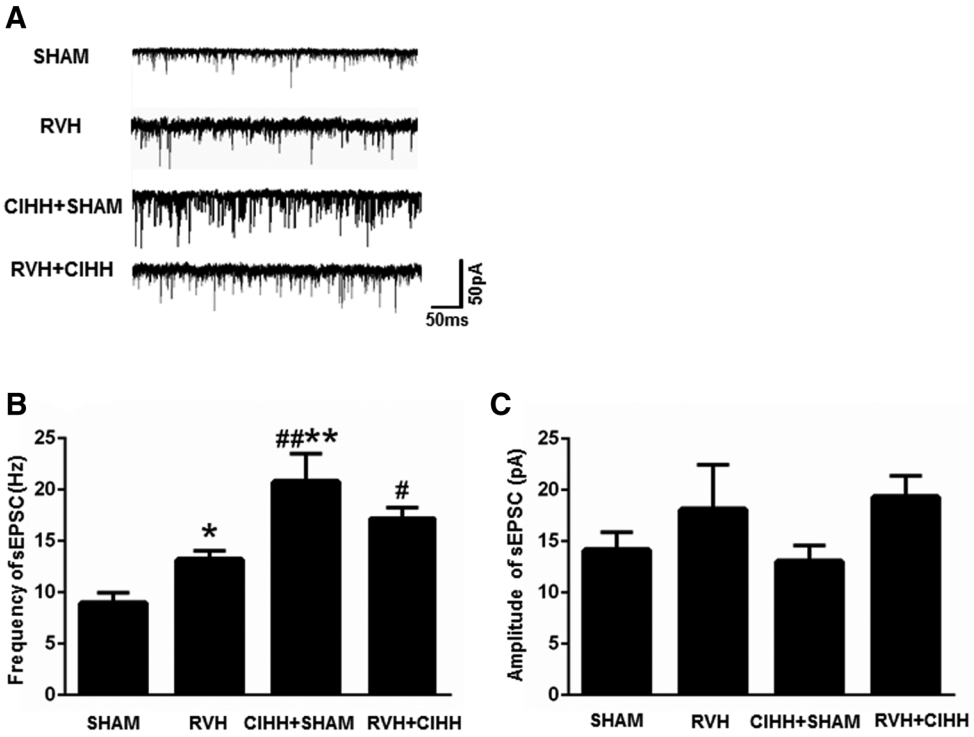

Fig. 6 Effect of CIHH on spontaneous excitatory postsynaptic currents (sEPSCs) in labeled NTS neurons. **A** Raw traces of sEPSCs in labeled NTS neurons. **B**, **C** Averaged frequency and amplitude of sEPSCs in labeled NTS neurons. SHAM, sham group, $n = 14$ NTS neurons; RVH, renal vascular hypertension group, $n = 10$ NTS neurons; CIHH + SHAM, chronic intermittent hypobaric hypoxia group, $n = 12$ NTS neurons; RVH + CIHH, RVH group receiving CIHH treatment, $n = 12$ NTS neurons. Data are expressed as mean ± SEM, $^{**}P < 0.01$ compared with SHAM group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with RVH group.

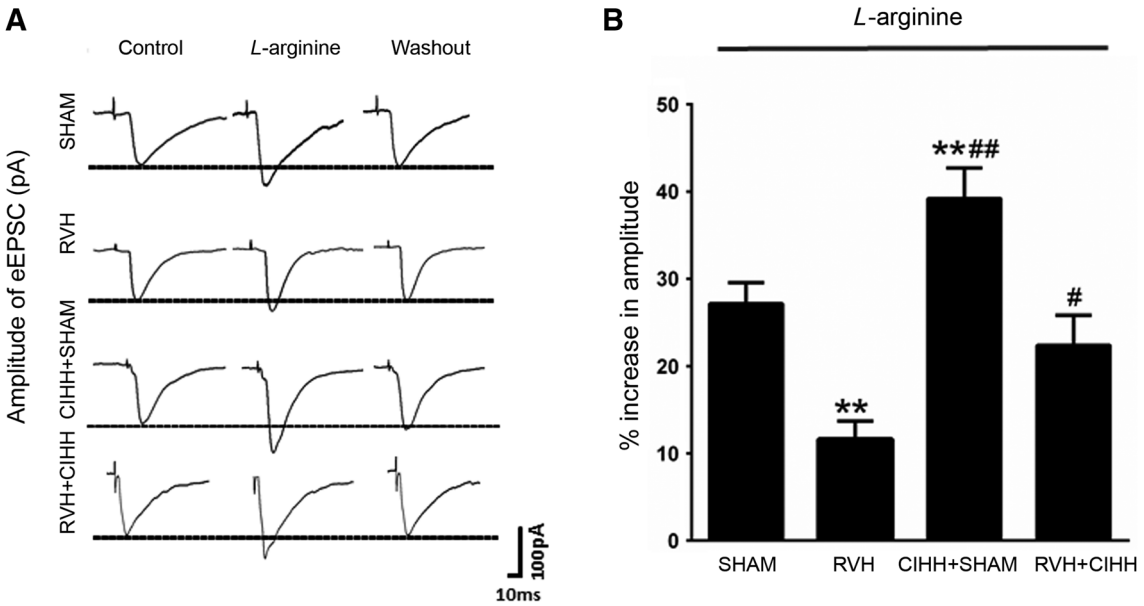

Fig. 7 Effect of *L*-arginine on eEPSCs in labeled NTS neurons. **A**, **B** Raw tracings of eEPSCs in labeled NTS neurons (**A**) and summary data of the percentage change of amplitude of eEPSCs (**B**) showing that the *L*-arginine-induced increase in the amplitude of eEPSCs was attenuated in RVH rats. CIHH treatment potentiated the *L*-arginine-induced increase in eEPSC amplitude and restored the attenuated response of eEPSCs to *L*-arginine. SHAM, sham group, $n = 21$ NTS neurons; RVH, renal vascular hypertension group, $n = 29$ NTS neurons; CIHH + SHAM, CIHH group, $n = 15$ NTS neurons; RVH + CIHH, RVH group receiving CIHH treatment, $n = 19$ NTS neurons. All data are expressed as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with SHAM group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with RVH group.

CIHH and RVH + CIHH rats than in RVH rats ($P < 0.05$, Fig. 9). These data indicate that CIHH treatment increases NO production by NOS in the NTS and improves the decreased NO production in hypertensive rats.

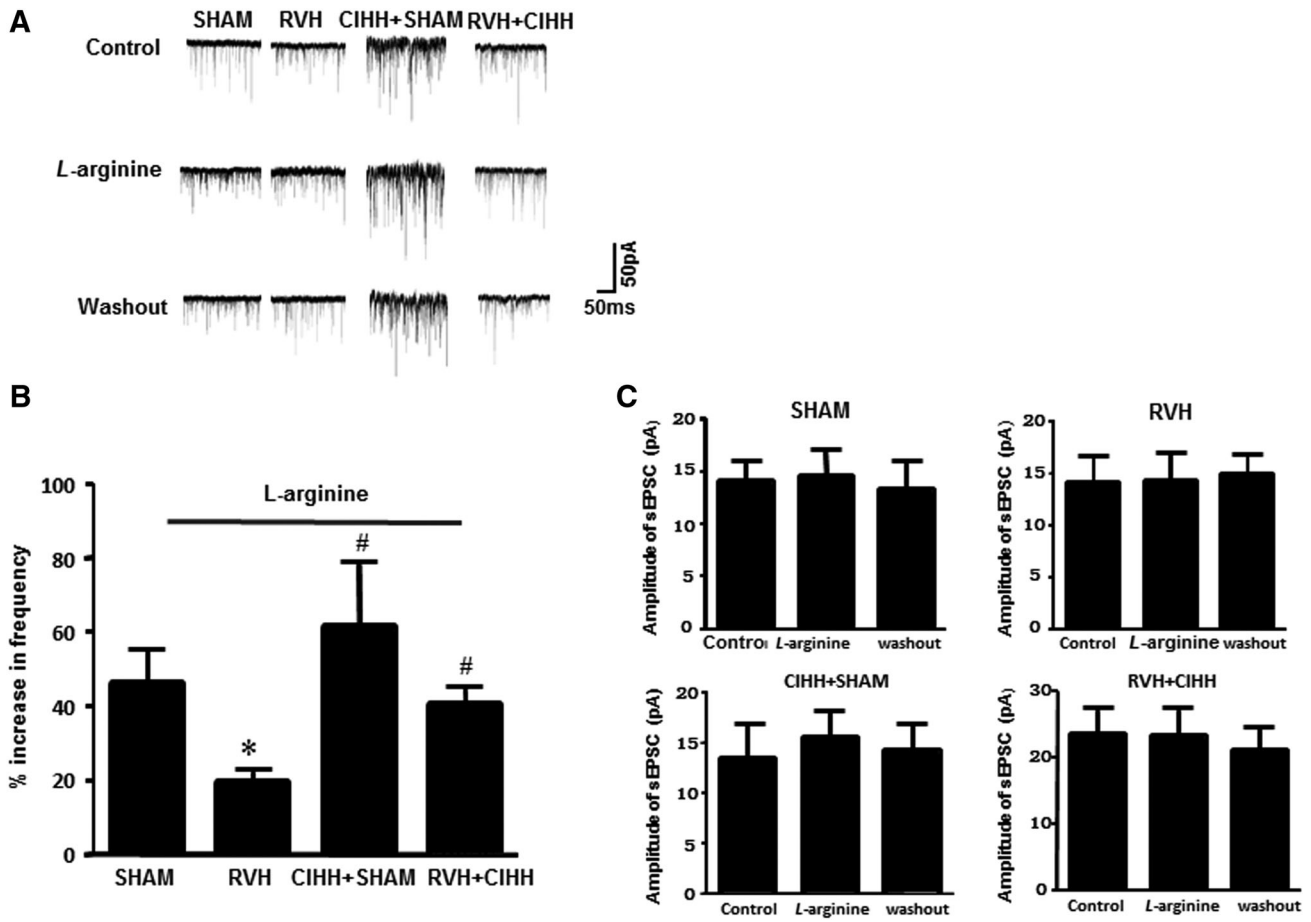

Fig. 8 Effect of *L*-arginine on sEPSCs in labeled NTS neurons. **A** Representative traces of sEPSCs in labeled NTS neurons. **B**, **C** Averaged frequency and amplitude of sEPSCs in labeled NTS neurons during application of 100 μmol/L *L*-arginine. SHAM, sham group, $n = 21$ NTS neurons; RVH, renal vascular hypertension group, $n = 19$ NTS neurons; CIHH + SHAM, chronic intermittent hypobaric hypoxia group, $n = 16$ NTS neurons; RVH + CIHH, RVH group receiving CIHH treatment, $n = 22$ NTS neurons. All data are expressed as mean ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with SHAM group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with RVH group.

Discussion

In this study, we investigated the central mechanism of CIHH-induced baroreflex facilitation by recording ABP, RSNA, and HR using electrophysiology, recording eEPSCs and sEPSCs using brain slice patch clamp, and assaying the expression of nNOS and eNOS proteins in the NTS by western blot. The results showed that CIHH treatment effectively reduced the ABP and potentiated the depressor effect induced by microinjection of the NO precursor *L*-arginine into the NTS in RVH rats. Furthermore, CIHH treatment increased the basal eEPSCs and augmented the *L*-arginine-induced increase in eEPSCs as well as the *L*-arginine-induced increase in sEPSCs frequency in NTS neurons receiving baroreceptor afferents in RVH rats. In addition, CIHH normalized the expression levels of nNOS and eNOS proteins in the NTS in RVH rats. These findings suggest that CIHH has an anti-hypertension effect in RVH rats, and this is related to the increase in nNOS and eNOS expression in the NTS and the potentiation of NO-induced excitatory synaptic inputs to the NTS neurons receiving baroreceptor afferents.

The NTS is the primary region receiving baroreceptor afferents and plays an important role in the regulation of ABP and sympathetic activity. Baroreceptor-sensitive NTS neurons are mainly distributed in the intermediate and caudal part of the NTS. These neurons receive baroreceptor afferents from the carotid sinus and aortic arch and project to another cardiovascular center after integrating the afferent information [18]. NTS lesions or pharmacological blockade of NTS baroreceptor afferent neurons leads to an acute increase in BP. In contrast, stimulation of the NTS by microinjection of the excitatory neurotransmitter glutamate induces a decrease in BP and bradycardia [18]. The antihypertensive effect of CIHH has been demonstrated in patients with essential hypertension and in spontaneously-hypertensive rats [4, 5]. Our previous studies have shown that CIHH facilitates the baroreceptor reflex and enhances

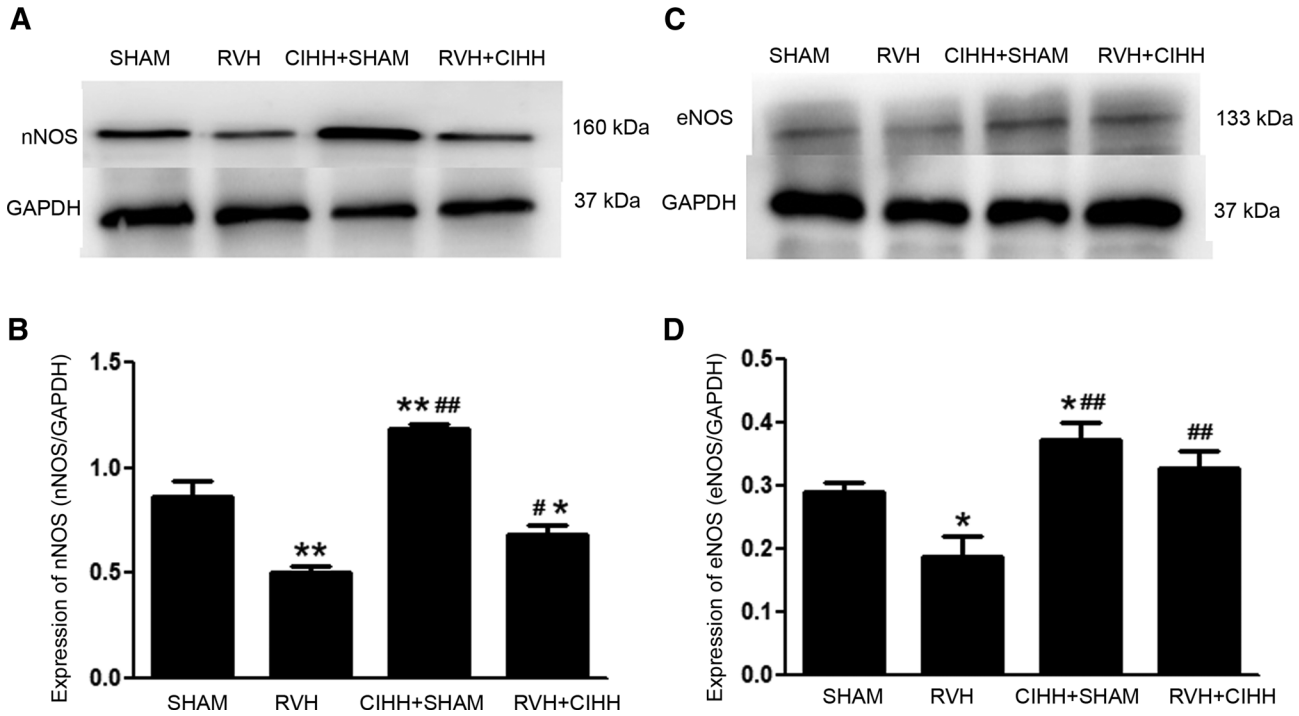

Fig. 9 Effect of CIHH on expression of nNOS and eNOS proteins in the NTS. **A**, **B** Original gel images (**A**) and summary data (**B**) showing that the nNOS protein level was higher in RVH rats receiving CIHH treatment than in RVH rats. **C**, **D** Gel images (**C**) and summary data (**D**) showing that the eNOS protein level was higher in RVH rats receiving CIHH than in RVH rats. Each group contains 4 samples and each sample contains NTS tissue from 1 rat. All data are expressed as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with SHAM group; $^{\#}P < 0.05$ $^{\#\#}P < 0.01$ compared with RVH group.

the stable reduction of fluctuations in BP [7]. Recently, we confirmed that CIHH enhances baroreflex sympathetic inhibition, facilitates the baroreceptor reflex, and antagonizes the impaired baroreceptor reflex function in renal hypertensive rats [6]. The present study further investigated the central mechanism of the CIHH anti-hypertension effect and focused on the NTS in RVH rats. We found that CIHH treatment significantly enhanced the baroreceptor sympathetic inhibition induced by the microinjection of *L*-arginine, and enhanced the electrical activity of the NTS baroreceptor-sensitive neurons. These results indicate that the facilitation of the baroreceptor reflex may be due to the enhancement of excitability in NTS neurons receiving baroreceptor afferents.

NO is an important signaling molecule that regulates many physiological functions, such as the regulation of cardiovascular activity under both physiological and pathophysiological conditions. eNOS and nNOS exist in the cell bodies and axons of NTS neurons and catalyze *L*-arginine to generate NO [19]. NO in the central nervous system contributes to regulation of the baroreceptor reflex and the decrease of NO production leads to hypertension [20]. NO in the NTS plays an important role in the regulation of sympathetic nerve activity, ABP, and HR. Although the action of NO has been extensively studied in the peripheral and central nervous systems, the role of NO in the NTS in cardiovascular regulation and baroreflex function is not fully understood. Also, the action and mechanism of NO in the central nervous system remain controversial [21–24]. For example, microinjection of the NO precursor *L*-arginine into the NTS reduces ABP [25], but intracerebroventricular injection of *L*-arginine causes a transient elevation of BP [26]. Blocking NO synthesis with *L*-N^G-monomethyl arginine citrate in the NTS produces dose-dependent responses of ABP and HR: a low dose reduces ABP, while a high dose increases it [24]. Consistent with previous studies showing that microinjection of *L*-arginine into the NTS induces a baroreflex-like response [27], we found that microinjection of *L*-arginine into the NTS decreased ABP, RSNA, and HR. This baroreflex-like sympathetic inhibitory response induced by *L*-arginine was attenuated in RVH rats. CIHH treatment not only enhanced the *L*-arginine-induced sympathetic inhibitory response but also restored the attenuated *L*-arginine-induced sympathetic inhibitory response in RVH rats. These data provide strong evidence that CIHH treatment decreases BP by enhancing baroreflex function. We also found that CIHH treatment significantly up-regulated the expression of nNOS and eNOS in the NTS in SHAM rats. Furthermore, CIHH restored the decreases in nNOS and

eNOS expression levels in the NTS in RVH rats. This up-regulation of nNOS and eNOS in the NTS may be involved in augmentation of the *L*-arginine-induced sympathetic inhibitory response by the CIHH treatment.

Previous studies have shown that the regulation of baroreflex function by NO is closely associated with glutamatergic neurotransmission in the NTS [28]. Glutamate acts on metabotropic and ionotropic receptors. Metabotropic glutamate receptors are G-protein-coupled receptors while ionotropic glutamate receptors are ligand-gated receptor channels, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-*D*-aspartate (NMDA), and kainite receptors. AMPA receptors mediate the fast components of glutamate signaling, while NMDA receptors mediate the slow and sustained components of glutamate signaling. These receptors are involved in regulating a variety of autonomic functions, such as the baroreflex and the cardiopulmonary reflex [29]. Microinjection of glutamate into the NTS induces a depressor effect and bradycardia, similar to the baroreceptor reflex [28]. Blocking ionotropic glutamate receptors with the antagonist kynurenic acid increases BP [30]. Previous studies have shown that glutamate is critically involved in the hypotensive response induced by an NO donor in the NTS [31]. Furthermore, NO donors increase NTS glutamatergic synaptic inputs through the soluble guanylate cyclase-cGMP signaling pathway [10]. Morphological data have shown that nNOS and glutamate immunoreactivity coexist in neuronal somata and axons in the NTS [32], providing additional evidence that NO interacts with glutamate in the NTS. NO increases glutamatergic synaptic inputs from primary afferents, including the baroreceptor afferents, because evoked EPSCs were elicited by stimulating the TS. Thus, these findings suggest that the NO-induced increase in glutamatergic synaptic input to NTS neurons is involved in NO-mediated baroreflex responses. The NO-induced increase in glutamatergic synaptic input to NTS neurons might be one of the mechanisms underlying the anti-hypertensive effect of CIHH treatment, which needs further investigation.

Many studies have demonstrated that CIHH has many beneficial effects, such as a protective effect on heart, brain, and liver [33], improvement of metabolic disturbance [34], and a regulatory effect on immune function [35]. Our previous study showed that 28 days of CIHH treatment (simulating 5000 m altitude, 6 h/day) clearly protects the heart against ischemia/reperfusion (I/R) injury and I/R-induced arrhythmia in rats [12]. Multiple mechanisms and signaling molecules have been proposed for the cardiovascular protection by CIHH, such as induction of heat-shock proteins [36], increased myocardial capillary angiogenesis and coronary flow [37], activation of ATP-sensitive K^+ channels, inhibition of mitochondrial permeability transition pores opening [38], and activation of NOS [11] and protein kinase C [39]. In addition, CIHH has been found to decrease the ABP in patients with essential hypertension and in spontaneously-hypertensive rats [4, 5, 40, 41]. Recently, our study demonstrated the anti-hypertensive effect of CIHH in RVH rats, which is associated with facilitation of the baroreflex [6]. In the present study, we confirmed the anti-hypertensive effect of CIHH again and determined the important role of NO in the NTS for facilitation of the baroreflex by CIHH treatment. Furthermore, CIHH treatment, due to its advantages of simplicity, ease of application, safety, and cost-effectiveness, is broadly applicable and has potentially important clinical use.

In summary, this study revealed that CIHH treatment up-regulated nNOS and eNOS proteins and increased NO-mediated synaptic glutamate release onto NTS neurons receiving baroreceptor afferents. Furthermore, CIHH treatment restored the decreased nNOS and eNOS expression levels and NO-mediated synaptic glutamate release onto NTS neurons receiving baroreceptor afferents in RVH rats. This information provides a rationale for developing novel therapeutics to treat renal hypertension.

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Conflict of interest No competing interests are declared by the authors.

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