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Inhibitory effects of sulfur dioxide within the nucleus tractus solitarius of rats: involvement of Calcium Ion channels, Adenine nucleoside triphosphate-sensitive potassium channels, and the nitric oxide/cyclic Guanine trinucleotide phosphate pathway

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This study was designed to investigate the cardiovascular effects of sulfur dioxide within the nucleus tractus solitarius. Sulfur dioxide or artificial cerebrospinal fluid was unilaterally applied into the nucleus tractus solitarius of rats, and the effects on blood pressure, heart rate, and arterial baroreflex sensitivity (ABR) were determined. To explore the mechanisms of the effects of intra-nucleus tractus solitarius sulfur dioxide, various inhibitors were applied prior to sulfur dioxide treatment. Unilateral microinjection of sulfur dioxide produced a dose-dependent decrease in blood pressure in anesthetized rats. Significant decreases in heart rate were also seen after unilateral microinjection of 20 and 200 pmol of sulfur dioxide ($P < 0.05$). Bilateral microinjection of sulfur dioxide into the nucleus tractus solitarius significantly decreased blood pressure and heart rate and also attenuated ABR. Pretreatment with glibenclamide or nicardipine within the nucleus tractus solitarius did not alter the hypotension or bradycardia ($P > 0.05$) induced by intra-nucleus tractus solitarius sulfur dioxide. Pretreatment with 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one, however, significantly attenuated this hypotension and bradycardia. Prior application of kynurenic acid or N(G)-Nitro-L-arginine

methyl ester into the nucleus tractus solitarius partially diminished the hypotension and bradycardia induced by intra-nucleus tractus solitarius sulfur dioxide. Our present study shows that sulfur dioxide produces cardiovascular inhibitory effects in the nucleus tractus solitarius, predominantly mediated by glutamate receptors and the nitric oxide/cyclic GMP signal transduction pathway. *NeuroReport* 30:914–920 Copyright © 2019 The Author(s). Published by Wolters Kluwer Health, Inc.

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Introduction

Sulfur dioxide (SO₂) is a major toxic gas and environmental pollutant [1]. Its toxicity has been extensively investigated in humans, animals, and plants [2]. SO₂, however, is endogenously produced during the metabolism of sulfur-containing amino acids in mammals [3]. It has also been shown to exert extensive physiological and pathological functions in cardiovascular system [3]. With a relative molecular mass of 64 g/mol, SO₂ can readily pass through the membrane independently of a specific receptor. On the basis of these studies, SO₂ has been proposed as a novel endogenous gaseous signaling molecule alongside nitric oxide (NO), carbon monoxide,

and hydrogen sulfide [4]. We have previously shown that aspartate aminotransferase (AAT), a key enzyme in endogenous SO₂ generation, is produced in the mammalian brain [5] and causes hypertension and tachycardia [6]. The central nervous system (CNS) is known to be involved in regulating arterial pressure [7–9]. The present study was designed to test the hypothesis that SO₂ regulates blood pressure (BP) by a central mechanism.

It is well known that the nucleus tractus solitarius (NTS) has a key role in regulating BP [10]. Neurons of the nucleus tractus solitarius (NTS) project directly or indirectly to structures of the medulla that regulate the activity of sympathetic and parasympathetic preganglionic neurons [11]. Although the NTS plays a key role in mediating tonic and reflex control of the cardiovascular system, the cardiovascular effects of SO₂ in the NTS have not been determined. Accordingly, the present study was designed to investigate the cardiovascular effects of intra-NTS SO₂,

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Materials and methods

Animals

Experiments were performed on 78 adult male Sprague–Dawley rats (2.5–3.0 kg) obtained from the Lanzhou University Laboratory Animal Center. Rats were housed under standard laboratory conditions, with a 12/12-h light/dark cycle and controlled temperature ($23 \pm 2^\circ\text{C}$). Animal treatment complied with the National Institutes of Health (NIH Publication 80-23) and our institute guidelines for the care and use of laboratory animals. Animal preparation, microinjection, and histological procedures were performed as described previously [12].

General procedures

Briefly, after 3 days of acclimatization, animals were anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneal injection (i.p.)) or combined anesthesia (800 mg/kg urethane, 40 mg/kg α -chloralose, and 40 mg/kg sodium tetraborate; i.p.). A trachea was then cannulated and connected to an animal ventilator (DW-2000; Shanghai Jiapeng Technology Co. Ltd, Shanghai, China) to facilitate mechanical respiration (10–12 ml/kg, 60–70 times/min). Except for those being measured for baroreflex sensitivity, rats were paralyzed with gallamine triethiodide (10 mg/kg initially and then 4 mg/kg every 30 min, intravenous injection (i.v.)) and artificially ventilated with oxygen-enriched room air. The right femoral artery and vein were then cannulated with polyethylene catheters to measure arterial BP and administer drugs, respectively. Mean arterial pressure (MAP) and heart rate (HR) were calculated using an RM6240 recording system (Chengdu Technology Co. Ltd, Chengdu, China). When necessary, supplemental doses of α -chloralose (20 mg/kg, i.v.) were administered to maintain an appropriate level of anesthesia. Subsequently, rats were fixed in a stereotaxic apparatus (MP-8003; RWD life Technology Co. Ltd, Shenzhen, China), and the dorsal surface of the medulla was surgically exposed. Body temperature was maintained at approximately 37°C with an infrared heating lamp.

Microinjection procedure

Coordinates for microinjections into the NTS were determined by stereotaxic atlas. A multi-barreled micropipette (tip diameter 20–30 μm) was inserted into the NTS (0.5–0.8 mm rostral to the obex, 0.5–1.0 mm lateral to the midline, and 0.2–0.5 mm below the dorsal surface of the medulla). The micropipette was filled with L-glutamate, SO_2 (2, 20, or 200 pmol), glibenclamide, nicardipine, N(G)-Nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4] Oxadiazolo[4,3-a] quinoxalin-1-one (ODQ), kynurenic acid (KYN), or artificial cerebrospinal fluid (aCSF) using a microsyringe. Glibenclamide, nicardipine, and ODQ were initially dissolved in dimethyl sulfoxide (DMSO) and then diluted to the final concentration with aCSF (133.3 mM sodium chloride, 3.4 mM potassium chloride, 1.3 mM calcium chloride, 1.2 mM Magnesium chloride, 0.6 mM Sodium dihydrogen phosphate, 32.0 mM Sodium

bicarbonate, and 3.4 mM glucose). pH was adjusted to 7.4 with 10% HCl. The final concentration of DMSO in aCSF was less than 1%, which produced little effect on BP or HR of rats in preliminary experiments. Other drugs were dissolved directly in aCSF. The selected drugs were based on preliminary experiments and previous study [12]. Drugs were prepared over a period of 5 to 10 s, and the injection volume (100 nl) was carefully measured by observing the movement of the fluid meniscus along a reticule under the guidance of an operating microscope. Each animal received only one microinjection into the NTS. Functional identification of the NTS was based on obtaining a depressor response to a microinjection of glutamate (2 nmol).

Determination of baroreflex sensitivity

After surgery, rats were stabilized for at least 30 min before baseline BP and HR measurements were taken. Baroreflex sensitivity was measured in rats given combined anesthesia, using a method described previously [12]. In brief, a bolus intravenous injection of phenylephrine (10 $\mu\text{g}/\text{kg}$) was administered to raise systolic BP (SBP) by 20 to 40 mmHg, before and 5 or 30 min after, bilaterally injecting SO_2 into the NTS (20 pmol each side). At least 10 min was allowed between phenylephrine injections. The relationship between heart beat period and SBP was analyzed by linear regression analysis (correlation coefficient, $R^2 > 0.8$) to determine ABR (arterial baroreflex reflex, heart beat period [ms]/SBP [mmHg]).

Histological analysis

After completing experiments, an overdose of urethane (0.4 g/kg, i.v.) was applied to deeply anesthetize the rats and 20 μl of 2% pontamine sky blue solution was injected to verify the microinjection sites. The brain stem was then removed and fixed for 48 to 72 h in a 10% paraformaldehyde–saline solution containing 30% sucrose. Subsequently, frozen brains were sectioned (50 μm) in the coronal view and stained with neutral red.

Statistical analysis

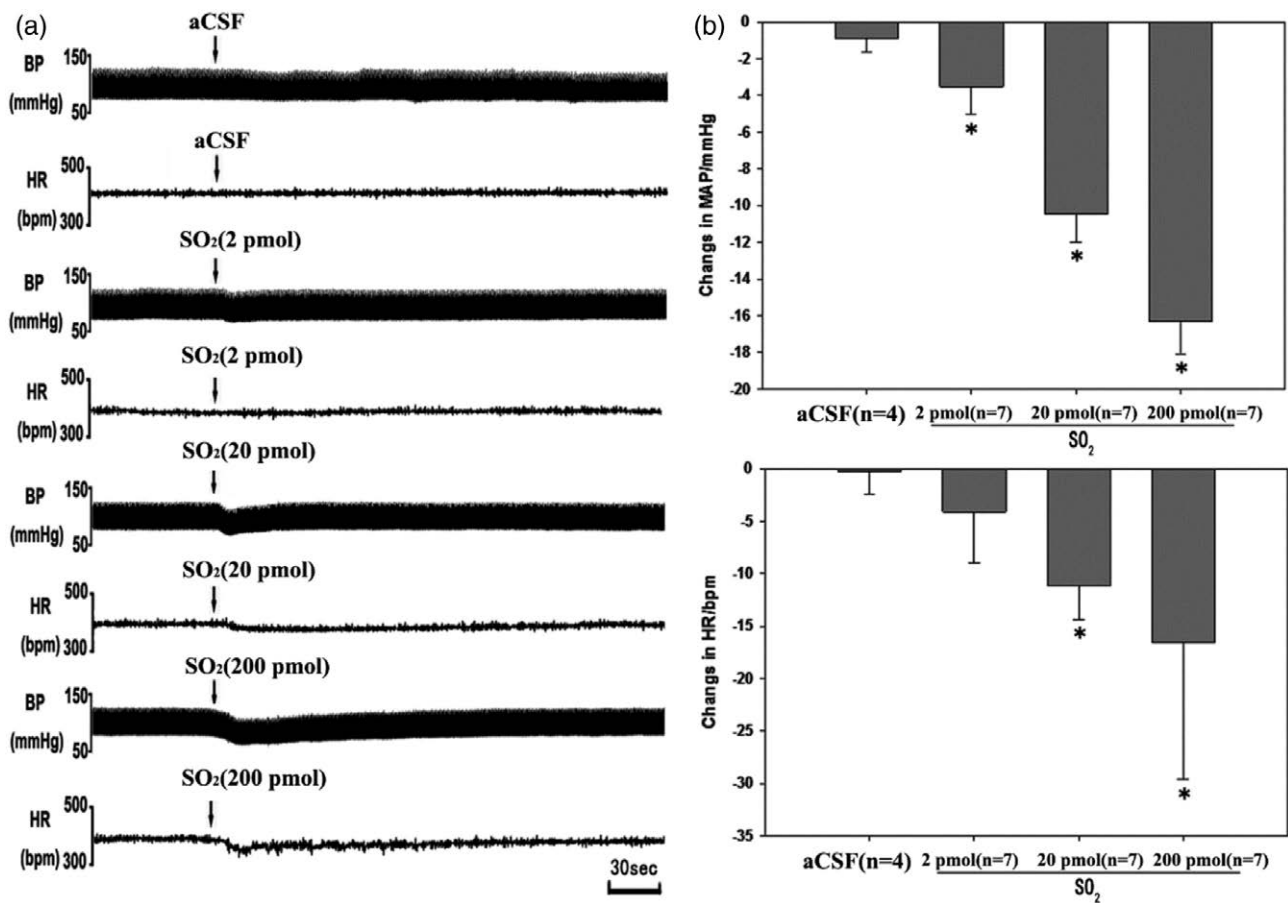
All values were expressed as mean \pm SEM. BP is presented as MAP, calculated from the following formula: diastolic BP + [(systolic – diastolic BP)/3]. MAP and HR before and after treatment were analyzed by repetitive-measure analysis of variance (ANOVA). Multiple groups' means were compared by one-way ANOVA and Newman–Keuls test. A value of $P < 0.05$ was considered statistically significant.

Results

Cardiovascular responses to microinjection of sulfur dioxide into the nucleus tractus solitarii

Figure 1a shows representative traces of BP and HR responses to the microinjection of SO_2 (2–200 pmol) or aCSF (100 nl) into the NTS. Intra-NTS injection of aCSF did not alter basal MAP [106 ± 16 vs 105 ± 16 mmHg, $F(1, 3) = 5.703$, $P > 0.05$] or HR [438 ± 23 vs 438 ± 24 bpm, $F(1, 3) = 0.000$, $P > 0.05$]. Topical application of SO_2 ,

Fig. 1



Topical application of SO₂-induced hypotension and bradycardia. (a) Representative original tracings showing the BP and HR response by unilateral microinjection of SO₂ (2–200 pmol) or aCSF (100 nl) into the NTS of rats; (b) Magnitude of changes in MAP and HR by unilateral microinjection of SO₂ (2–200 pmol) or aCSF (100 nl) into the NTS (mean ± SEM). **P* < 0.05 vs vehicle (aCSF). aCSF, artificial cerebrospinal fluid; BP, blood pressure; HR, heart rate; MAP, mean arterial pressure; NTS, nucleus tractus solitarii; SO₂, aqueous solution of sulfur dioxide.

however, produced dose-dependent hypotension (2 pmol: -4 ± 1 mmHg; 20 pmol: -10 ± 2 mmHg; 200 pmol: -16 ± 2 mmHg) in anesthetized rats [*F* (1, 21) = 635.936, *P* < 0.05, compared with microinjection of aCSF: -1 ± 1 mmHg]. Although microinjection of a low dose of SO₂ (2 pmol) into the NTS did not significantly influence HR [-4 ± 5 bpm; *F* (1, 9) = 2.475, *P* > 0.05, compared with microinjection of aCSF: 0 ± 3 bpm], microinjection of higher doses (20 and 200 pmol) produced significant bradycardia [20 pmol: -11 ± 3 bpm; 200 pmol: -17 ± 13 bpm vs aCSF: 0 ± 3 bpm; *F* (1, 15) = 19.506, *P* < 0.05]. Hypotension and bradycardia occurred 5 s after topical application of SO₂, reached their nadir after 20 s, and returned to baseline levels after approximately 2 min. The cardiovascular responses to microinjection of aCSF and SO₂ are summarized in Fig. 1b.

Effects of intra-nucleus tractus solitarii sulfur dioxide microinjections on ABR

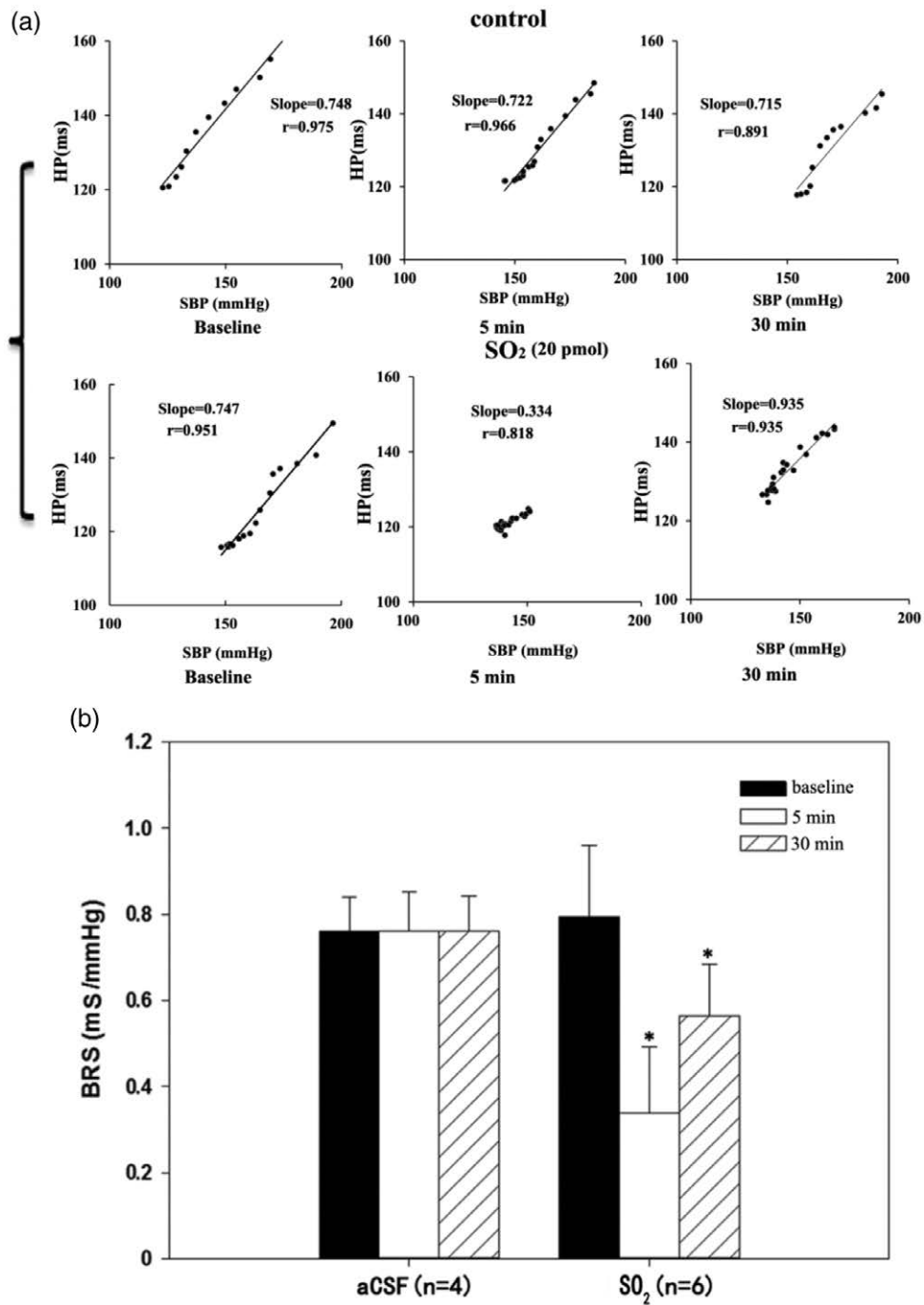
Fig. 2a and b shows the effects of the phenylephrine-evoked baroreflex before, 5 min after, and 30 min

after bilateral microinjection of SO₂ into the NTS. Bilateral microinjection of the vehicle, aCSF, did not alter basal ABR [5 min: 0.762 ± 0.091 ms/mmHg; 30 min: 0.760 ± 0.083 ms/mmHg vs control: 0.761 ± 0.078 ms/mmHg; *F* (2, 6) = 0.033, *P* > 0.05]. Bilateral microinjection of SO₂ into the NTS, however, significantly decreased basal MAP [from 105 ± 12 to 93 ± 12 mmHg, *F* (1, 6) = 336.940, *P* < 0.05] and HR [from 430 ± 25 to 416 ± 27 bpm, *F* (1, 6) = 82.964, *P* < 0.05] and attenuated ABR [5 min: 0.338 ± 0.154 ms/mmHg; 30 min: 0.564 ± 0.120 ms/mmHg vs control: 0.795 ± 0.166 ms/mmHg; *F* (2, 18) = 89.141, *P* < 0.05]. The effects of intra-NTS microinjection of SO₂ and aCSF on ABR are summarized in Fig. 2.

Effects of ATP-sensitive potassium blockade, L-type calcium channel blockade, and soluble guanylyl cyclase inhibition on cardiovascular functions affected by intra-nucleus tractus solitarii sulfur dioxide

Table 1 summarized the BP and HR responses to intra-NTS SO₂ following pretreatment with the ATP-sensitive

Fig. 2



The effects of bilateral microinjection of SO₂ on BP and HR responses induced by phenylephrine. (a) The sample traces of phenylephrine-evoked baroreflex before and after 5 and 30 min of microinjection of SO₂ (20 pmol for each side, n=6) or vehicle (aCSF, 100 nl for each side, n=4) into the NTS. Values of slope are the values of baroreflex sensitivity. (b) Responses of BRS before and after 5 min, 30 min of microinjection of SO₂ (20 pmol), or aCSF (100 nl) into the NTS. aCSF, artificial cerebrospinal fluid; BP, blood pressure; BRS, baroreflex sensitivity; HR, heart rate; HP, heart beat period; NTS, nucleus tractus solitarii; SBP, systolic blood pressure; SO₂, aqueous solution of sulfur dioxide.

potassium (K_{ATP}) blocker, glibenclamide, the L-type calcium channel blocker, nifedipine, and the soluble guanylyl cyclase (sGC) inhibitor, ODQ. In vehicle, prior

microinjection of the vehicle, consisting of aCSF in 1% DMSO, did not alter basal MAP [108 ± 4 vs 109 ± 5 mmHg, *F* (1, 3)=0.045, *P*>0.05] or HR [413 ± 42 vs

Table 1 The blood pressure and heart rate responses to intra-nucleus tractus solitarii sulfur dioxide following pretreatment with the following inhibitors

	n	ΔMAP (mmHg)	ΔHR (bpm)
Vehicle (aCSF in 1% DMSO, 100nl)+SO ₂ (20 pmol)	4	-10 ± 1	-13 ± 6
Glibenclamide (40nmol)+SO ₂ (20 pmol)	7	-9 ± 2	-10 ± 2
Nicardipine (200pmol)+SO ₂ (20 pmol)	7	-10 ± 3	-11 ± 6
ODQ (250pmol)+SO ₂ (20 pmol)	7	-3 ± 1 ^a	-3 ± 3 ^a
Vehicle (aCSF)+SO ₂ (20 pmol)	4	-10 ± 3	-14 ± 5
KYN (15 nmol)+SO ₂ (20 pmol)	7	-5 ± 2 ^a	-7 ± 2 ^a
L-NAME (15 nmol)+SO ₂ (20 pmol)	7	-6 ± 1 ^a	-8 ± 4 ^a

aCSF, artificial cerebrospinal fluid; DMSO, dimethyl sulfoxide; KYN, kynurenic acid; L-NAME, N(G)-Nitro-L-arginine methyl ester; MAP, mean arterial pressure; ODQ, 1H-[1,2,4]Oxadiazolo[4,3-a] quinoxalin-1-one; SO₂, sulfur dioxide.

^aP<0.05, compared with pretreatment with vehicle.

414 ± 42 bpm, $F(1, 3)=0.033$, $P>0.05$] and did not affect the hypotension and bradycardia induced by intra-NTS SO₂. Microinjection of glibenclamide into the NTS transiently decreased basal MAP [from 107 ± 5 to 103 ± 5 mmHg, $F(1, 3)=67.079$, $P<0.05$], but did not significantly alter basal HR [from 403 ± 27 to 398 ± 27, $F(1, 6)=3.102$, $P>0.05$]. Pretreatment with glibenclamide did not affect the hypotensive [-9 ± 2 mmHg pretreatment with glibenclamide vs -10 ± 1 mmHg pretreatment with aCSF, $F(1, 9)=0.105$, $P>0.05$] or bradycardic [-10 ± 2 bpm pretreatment with glibenclamide vs -13 ± 6 bpm pretreatment with aCSF, $F(1, 9)=0.133$, $P>0.05$] responses to SO₂ within the NTS. Microinjection of nicardipine into the NTS transiently decreased basal MAP [from 103 ± 11 to 97 ± 10 mmHg, $F(1, 6)=30.048$, $P<0.05$] and basal HR [from 411 ± 39 to 404 ± 39, $F(1, 6)=0.072$, $P<0.05$]. Pretreatment with nicardipine, however, did not affect the hypotensive [-10 ± 3 mmHg pretreatment with nicardipine vs -10 ± 1 mmHg pretreatment with aCSF, $F(1, 9)=0.700$, $P>0.05$] or bradycardic [-11 ± 6 bpm pretreatment with nicardipine vs -13 ± 6 bpm pretreatment with aCSF, $F(1, 9)=0.001$, $P>0.05$] responses to SO₂. Microinjection of ODQ into the NTS significantly decreased basal MAP [from 103 ± 11 to 90 ± 10 mmHg, $F(1, 6)=35.993$, $P<0.05$] and basal HR [from 413 ± 46 to 398 ± 46, $F(1, 6)=11.024$, $P<0.05$]. It also decreased the hypotension [-3 ± 1 mmHg pretreatment with ODQ vs -10 ± 1 mmHg pretreatment with aCSF, $F(1, 9)=96.814$, $P<0.05$] and bradycardia [-3 ± 3 bpm pretreatment with ODQ vs -13 ± 6 bpm pretreatment with aCSF, $F(1, 9)=14.431$, $P<0.05$] induced by intra-NTS SO₂. These results are summarized in Fig. 1 and Table 1.

Effects of glutamate receptor antagonism and nitric oxide synthase inhibition on the cardiovascular functions affected by intra-nucleus tractus solitarii sulfur dioxide

Table 1 summarized BP and HR responses to intra-NTS SO₂ following pretreatment with the glutamate receptor antagonist, KYN, and the non-specific NO

synthase inhibitor, L-NAME. Prior microinjection of aCSF did not alter basal MAP [110 ± 7 vs 109 ± 8 mmHg, $F(1, 3)=0.186$, $P>0.05$] or HR [407 ± 32 vs. 406 ± 42 bpm, $F(1, 3)=0.125$, $P>0.05$] and did not affect the hypotension and bradycardia induced by intra-NTS SO₂. Microinjection of KYN into the NTS significantly increased basal MAP [from 109 ± 8 to 115 ± 9 mmHg, $F(1, 6)=26.528$, $P<0.05$] and basal HR [from 415 ± 44 to 422 ± 43, $F(1, 6)=8.132$, $P<0.05$] and significantly decreased the hypotensive [-5 ± 2 mmHg pretreatment with KYN vs -10 ± 3 mmHg pretreatment with aCSF, $F(1, 9)=12.814$, $P<0.05$] and bradycardic [-7 ± 2 bpm pretreatment with KYN vs -14 ± 5 bpm pretreatment with aCSF, $F(1, 9)=7.998$, $P<0.05$] responses of SO₂ to intra-NTS SO₂. Microinjection of L-NAME into the NTS significantly decreased basal MAP [from 102 ± 10 to 92 ± 10 mmHg, $F(1, 6)=65.006$, $P<0.05$] and basal HR [from 429 ± 52 to 416 ± 53, $F(1, 6)=41.041$, $P<0.05$] and significantly decreased the hypotension [-6 ± 1 mmHg pretreatment with L-NAME vs -10 ± 3 mmHg pretreatment with aCSF, $F(1, 9)=16.716$, $P<0.05$] and bradycardia [-8 ± 4 bpm pretreatment with L-NAME vs -14 ± 5 bpm pretreatment with aCSF, $F(1, 9)=11.755$, $P<0.05$] induced by intra-NTS SO₂. These results are summarized in Table 1.

Discussion

In this study, we show that the intra-NTS application of SO₂ produces dose-dependent hypotension and bradycardia in anesthetized rats, which are most likely mediated by activation of the NO/cyclic GMP (cGMP) signal transduction pathway and/or glutamate receptors. Furthermore, bilateral injection of SO₂ into the NTS was found to significantly reduce BP and HR and attenuate ABR at the NTS level.

SO₂ has been traditionally viewed as a toxic gas and a serious environmental pollutant. It is, however, also produced endogenously from the metabolism of sulfur-containing amino acids in mammals [13]. The cardiovascular effects of SO₂ have been extensively studied and include antihypertension, vasodilation, amelioration of vascular remodeling, antioxidative capacities, regulation of lipid metabolism, and intracellular signal transduction [14]. A previous study has shown that SO₂ and the key enzyme, AAT-1, are distributed widely in the CNS [15]. As a small gas molecule, SO₂ can also pass into the CNS readily through the blood-brain barrier. The present study was designed to test the hypothesis that SO₂ influences cardiovascular function by a central mechanism and to investigate the details of this mechanism.

As the first projection site of afferent fibers from arterial baroreceptors and chemoreceptors, the NTS is known to be important in maintaining cardiovascular autonomic and visceral stability [10]. The mechanisms, however, involved in SO₂-mediated cardiovascular effects in the NTS remain unclear. The present study aimed to explore these mechanisms.

Here, we showed that microinjection of SO_2 into the NTS produces dose-dependent cardiovascular inhibitory effects, similar to those seen after microinjection of the excitatory amino acid, L-glutamate. L-glutamate is the primary neurotransmitter released from sensory afferents, and its microinjection into the NTS of anesthetized rats reduces arterial pressure [16]. We hypothesized that the cardiovascular effects caused by intra-NTS SO_2 are mediated by glutamate receptors. We found, however, that the cardiovascular effects of intra-NTS SO_2 are only partly decreased by pretreatment with KYN. Therefore, these effects may be mediated by alternative mechanisms. Activation of glutamate receptors within the NTS has been shown to enhance ABR in rats [17]. We found that bilateral microinjection of SO_2 into the NTS inhibited ABR, which indirectly indicates that the cardiovascular effects of intra-NTS SO_2 are not completely mediated by glutamate receptors.

SO_2 has been shown to produce dose-dependent vasorelaxing effects in the peripheral cardiovascular system. At low SO_2 concentration ($<450 \mu\text{mol/l}$), vasorelaxation involves the big-conductance calcium ion (Ca^{2+})-activated K^+ (BKCa) channel, whereas at high SO_2 concentration ($>500 \mu\text{mol/l}$), it is associated with K_{ATP} channel activation and the L-type calcium channel (L-Ca^{2+}) channel [18]. Furthermore, previous studies using SO_2 derivatives have shown that the relaxing effect of SO_2 is related to the Prostacyclin-adenylyl cyclase-cyclic adenosine 3',5'-monophosphate protein-kinase A (PGI_2 -AC-cAMP-PKA) signaling pathway [19]. Hence, ion channels, such as L-Ca^{2+} , K_{ATP} , and BKCa channels, as well as cGMP and cAMP pathways, play important roles in the effects of SO_2 on vasodilation. Pharmacological studies have shown that K_{ATP} channels, L-type calcium channels, and NO are widely distributed in the NTS [20,21]. These reports led us to speculate that the cardiovascular functions of intra-NTS SO_2 may be mediated by K_{ATP} channels, L-type calcium channels, or NO. To test this hypothesis, we microinjected the K_{ATP} channel blocker, glibenclamide, the L-type calcium channel blocker, nifedipine, or the nonselective inhibitor of NO synthetases, L-NAME, prior to microinjection of SO_2 . Neither glibenclamide nor nifedipine significantly influenced the cardiovascular effects of intra-NTS SO_2 . This suggests that the mechanism whereby intra-NTS SO_2 induced hypotension and bradycardia did not involve K_{ATP} channels or L-type calcium channels. Pretreatment with L-NAME, however, significantly decreased the hypotension and bradycardia induced by intra-NTS injection of SO_2 . Furthermore, the NO-sensitive guanylyl cyclase inhibitor, ODO, almost abolished the hypotension and bradycardia induced by intra-NTS injection of SO_2 . It has been reported that SO_2 -induced relaxation of phenylephrine-precontracted rat aortic rings is mediated by endothelial nitric oxide synthase [22]. Our present study also suggested that the hypotension and bradycardia induced by intra-NTS SO_2

are mediated by the activation of the NO/cGMP signal transduction pathway and/or glutamate receptors.

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Conflicts of interest

There are no conflicts of interest.

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