Inhibitory effects of sulfur dioxide within the nucleus tractus solitarii 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 solitarii. Sulfur dioxide or artificial cerebrospinal fluid was unilaterally applied into the nucleus tractus solitarii 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 solitarii 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 solitarii significantly decreased blood pressure and heart rate and also attenuated ABR. Pretreatment with glibenclamide or nicardipine within the nucleus tractus solitarii did not alter the hypotension or bradycardia (P>0.05) induced by intra-nucleus tractus solitarii sulfur dioxide. Pretreatment with 1H-[1,2,4]Oxadiazolo[4,3-a]quinolinal-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 solitarii partially diminished the hypotension and bradycardia induced by intra-nucleus tractus solitarii sulfur dioxide. Our present study shows that sulfur dioxide produces cardiovascular inhibitory effects in the nucleus tractus solitarii, predominantly mediated by glutamate receptors and the nitric oxide/cyclic GMP signal transduction pathway.

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Introduction

Sulfur dioxide (SO\textsubscript{2}) is a major toxic gas and environmental pollutant [1]. Its toxicity has been extensively investigated in humans, animals, and plants [2]. SO\textsubscript{2}, 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, SO\textsubscript{2} can readily pass through the membrane independently of a specific receptor. On the basis of these studies, SO\textsubscript{2} 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 SO\textsubscript{2} 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 SO\textsubscript{2} regulates blood pressure (BP) by a central mechanism.

It is well known that the nucleus tractus solitarii (NTS) has a key role in regulating BP [10]. Neurons of the nucleus tractus solitarii (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 SO\textsubscript{2} in the NTS have not been determined. Accordingly, this present study was designed to investigate the cardiovascular effects of intra-NTS SO\textsubscript{2}.
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 ± 2°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 anesthe-
tized 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 polyethyl-
ene 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 deter-
mined by stereotaxic atlas. A multi-barreled micropi-
pette (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, 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 (13.35 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 dis-
solved directly in aCSF. The selected drugs were based on preliminary experiments and previous study [12]. Drugs were prepared over a period of 5 to 10s, 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 identi-
fication 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 com-
bined anesthesia, using a method described previously [12]. In brief, a bolus intravenous injection of phenylephrine (10 μg/kg) was administered to raise systolic BP (SBP) by 20 to 40 mmHg, before and 5 or 30 min after, bilaterally injecting SO₂ 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 (cor-
relation 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 ± 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 analy-
ysis of variance (ANOVA). Multiple groups' means were com-
pared 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 SO₂ (2–200 pmol) 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 SO₂,

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however, produced dose-dependent hypotension (2 pmol: $-4 \pm 1$ mmHg; 20 pmol: $-10 \pm 2$ mmHg; 200 pmol: $-16 \pm 2$ mmHg) in anesthetized rats [$F(1, 21) = 635.936$, $P < 0.05$, compared with microinjection of aCSF: $-1 \pm 1$ mmHg]. Although microinjection of a low dose of SO$_2$ (2 pmol) into the NTS did not significantly influence HR [$-4 \pm 5$ bpm; $F(1, 9) = 2.475$, $P > 0.05$, compared with microinjection of aCSF: $-0 \pm 2$ bpm], microinjection of higher doses (20 and 200 pmol) produced significant bradycardia [20 pmol: $-11 \pm 3$ bpm; 200 pmol: $-17 \pm 13$ bpm vs aCSF: $0 \pm 3$ bpm; $F(1, 15) = 19.506$, $P < 0.05$]. Hypotension and bradycardia occurred 5 s after topical application of SO$_2$, reached their nadir after 20 s, and returned to baseline levels after approximately 2 min. The cardiovascular responses to microinjection of aCSF and SO$_2$ 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 SO$_2$ into the NTS. Bilateral microinjection of the vehicle, aCSF, did not alter basal ABR [5 min: $0.762 \pm 0.091$ ms/mmHg; 30 min: $0.760 \pm 0.083$ ms/mmHg vs control: $0.761 \pm 0.078$ ms/mmHg; $F(2, 6) = 0.033$, $P > 0.05$]. Bilateral microinjection of SO$_2$ into the NTS, however, significantly decreased basal MAP [from $105 \pm 12$ to $93 \pm 12$ mmHg, $F(1, 6) = 336.940$, $P < 0.05$] and HR [from $430 \pm 25$ to $416 \pm 27$ bpm, $F(1, 6) = 82.964$, $P < 0.05$] and attenuated ABR [5 min: $0.338 \pm 0.154$ ms/mmHg; 30 min: $0.564 \pm 0.120$ ms/mmHg vs control: $0.795 \pm 0.166$ ms/mmHg; $F(2, 18) = 89.141$, $P < 0.05$]. The effects of intra-NTS microinjection of SO$_2$ 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 SO$_2$ following pretreatment with the ATP-sensitive

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Topical application of SO$_2$-induced hypotension and bradycardia. (a) Representative original tracings showing the BP and HR response by unilateral microinjection of SO$_2$ (2–200 pmol) or aCSF (100 nl) into the NTS of rats; (b) Magnitude of changes in MAP and HR by unilateral microinjection of SO$_2$ (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; SO$_2$, aqueous solution of sulfur dioxide.
potassium (K\textsubscript{ATP}) blocker, glibenclamide, the 1-type calcium channel blocker, nicardipine, 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...
The blood pressure and heart rate responses to intranucleus tractus solitarii sulfur dioxide following pretreatment with the following inhibitors

| Inhibitor                        | n | ΔMAP (mmHg) | ΔHR (bpm) |
|----------------------------------|---|-------------|-----------|
| Vehicle (aCSF in 1% DMSO, 100 nl) | 4 | -10 ± 1     | -13 ± 6   |
| Glucosamine (40 nmol) + SO_2 (20 pmol) | 7 | -9 ± 2      | -10 ± 2   |
| Nicardipine (200 pmol) + SO_2 (20 pmol) | 7 | -10 ± 3     | -11 ± 6   |
| ODO (20 pmol) + SO_2 (20 pmol) | 7 | -3 ± 1 ⁠*  | -3 ± 3   |
| Vehicle (aCSF) + SO_2 (20 pmol) | 4 | -10 ± 3     | -14 ± 5   |
| KYN (15 nmol) + SO_2 (20 pmol) | 7 | -5 ± 2 ⁠*  | -7 ± 2   |
| l-NAME (15 nmol) + SO_2 (20 pmol) | 7 | -6 ± 1 ⁠*  | -8 ± 4 ⁠*  |

aCSF, artificial cerebrospinal fluid; DMSO, dimethyl sulfoxide; KYN, kynurenic acid; l-NAME, N(G)-Nitro-l-arginine methyl ester; MAP, mean arterial pressure; ODO, 1H-[1,2,4]Oxadiazolo[4,3-a] quinoxalin-1-one; SO_2, sulfur dioxide.

*P<0.05, compared with pretreatment with vehicle.

Discussion

In this study, we show that the intra-NTS application of SO_2 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 SO_2 into the NTS was found to significantly reduce BP and HR and attenuate ABR at the NTS level.

SO_2 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 SO_2 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 SO_2 and the key enzyme, AAT-1, are distributed widely in the CNS [15]. As a small gas molecule, SO_2 can also pass into the CNS readily through the blood–brain barrier. The present study was designed to test the hypothesis that SO_2 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 SO_2-mediated cardiovascular effects in the NTS remain unclear. The present study aimed to explore these mechanisms.
Here, we showed that microinjection of SO₂ 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 SO₂ are mediated by glutamate receptors. We found, however, that the cardiovascular effects of intra-NTS SO₂ are 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 SO₂ into the NTS inhibited ABR, which indirectly indicates that the cardiovascular effects of intra-NTS SO₂ are not completely mediated by glutamate receptors.

SO₂ has been shown to produce dose-dependent vasorelaxing effects in the peripheral cardiovascular system. At low SO₂ concentration (<450 µmol/l), vasorelaxation involves the big-conductance calcium ion (Ca²⁺)-activated K⁺ (BKCa) channel, whereas at high SO₂ concentration (>500 µmol/l), it is associated with K ATP channel activation and the L-type calcium channel (L-Ca²⁺) channel [18]. Furthermore, previous studies using SO₂ derivatives have shown that the relaxing effect of SO₂ is related to the Prostacyclin-adenyl cyclase-cyclic adenosine 3',5'-monophosphate protein-kinase A (PGI₂-AC-cAMP-PKA) signaling pathway [19]. Hence, ion channels, such as L-Ca²⁺, K ATP, and BKCa channels, as well as cGMP and cAMP pathways, play important roles in the effects of SO₂ on vasodilatation. 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 SO₂ 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, nicardipine, or the nonselective inhibitor of NO synthetases, L-NAME, prior to microinjection of SO₂. Neither glibenclamide nor nicardipine significantly influenced the cardiovascular effects of intra-NTS SO₂. This suggests that the mechanism whereby intra-NTS SO₂ 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 SO₂. Furthermore, the NO-sensitive guanylyl cyclase inhibitor, ODQ, almost abolished the hypotension and bradycardia induced by intra-NTS injection of SO₂. It has been reported that SO₂-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 SO₂ 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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