Hydrogen sulfide (H2S) is now recognized as an important signaling molecule and has been shown to have vasodilator and cardio-protectant effects. More recently it has been suggested that H2S may also act within the brain to reduce blood pressure (BP). In the present study we have demonstrated the presence of the H2S-producing enzyme, cystathionine-β-synthase (CBS) in the rostral ventrolateral medulla (RVLM), and the hypothalamic paraventricular nucleus (PVN), brain regions with key cardiovascular regulatory functions. The cardiovascular role of H2S was investigated by determining the BP, heart rate (HR), and lumbar sympathetic nerve activity (LSNA) responses elicited by a H2S donor sodium hydrogen sulfide (NaHS) or inhibitors of CBS, microinjected into the RVLM and PVN. In anesthetized Wistar Kyoto rats bilateral microinjections of NaHS (0.2–2000 pmol/side) into the RVLM did not significantly affect BP, HR, or LSNA, compared to vehicle. Similarly, when the CBS inhibitors, amino-oxyacetate (AOA; 0.1–1.0 nmol/side) or hydroxylamine (HA; 0.2–2.0 nmol/side), were administered into the RVLM, there were no significant effects on the cardiovascular variables compared to vehicle. Microinjections into the PVN of NaHS, HA, and AOA had no consistent significant effects on BP, HR, or LSNA compared to vehicle. We also investigated the cardiovascular responses to NaHS microinjected into the RVLM and PVN in spontaneously hypertensive rats. Again, there were no significant effects on BP, HR, and LSNA. Together, these results suggest that H2S in the RVLM and PVN does not have a major role in cardiovascular regulation.

Keywords: hydrogen sulfide, paraventricular nucleus, rostral ventrolateral medulla, lumbar sympathetic nerve activity, cardiovascular
Whether NaHS microinjected into the RVLM can influence SNA to 

Activation of the PVN can elicit increases or decreases in SNA and 

Therefore, the aim of whether H2S acting in those brain regions has different effects in 

emanate from the PVN.

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artery were cannulated as described previously (Chen et al., 2008). Subsequently filled with the gas. Once anesthesia was induced, isoflurane (1–3% in 

SURGICAL PROCEDURES

The animals were housed in a temperature-controlled room on a 12:12 h light/dark cycle (lights on at 7:00 a.m.), in the RMIT Animal Facility (RMIT University, Bundoora West campus, VIC, Australia). The animals were housed for a minimum period of 1 week before undergoing any experimental procedure. All procedures were performed to conform to the guidelines set out by the National Health and Medical Research Council of Australia and were approved by the RMIT University Animal Ethics committee.

MATERIALS AND METHODS

ANIMALS

Male wistar kyoto (WKY) and spontaneously hypertensive (SHR) rats, weighing 300–350 g, were obtained from the Animal Resources Centre (ARC, Canning Vale, WA, Australia). The animals were housed in a temperature-controlled room on a 12:12 h light/dark cycle (lights on at 7:00 a.m.), in the RMIT Animal Facility (RMIT University, Bundoora West campus, VIC, Australia). The animals were housed for a minimum period of 1 week before undergoing any experimental procedure. All procedures were performed to conform to the guidelines set out by the National Health and Medical Research Council of Australia and were approved by the RMIT University Animal Ethics committee.

SURGICAL PROCEDURES

Rats were anesthetized initially with inhaled isoflurane (1–3% in air), by placing the animal into a sealed container which was subsequently filled with the gas. Once anesthesia was induced, isoflurane was continually administered via a mask while the femoral vein and artery were cannulated as described previously (Chen et al., 2008). Briefly, the right femoral vein and artery were exposed by blunt dissection and vein and artery were cannulated separately using two polyethylene catheters consisting of PE 10 tubing connected to PE 50 tubing, filled with heparinized saline (50 U/ml). Anesthesia was then maintained using urethane (1–1.5 g/kg iv) with supplemental doses as required (0.1–0.3 g/kg iv), administered through the cannulated vein. The depth of anesthesia was maintained to ensure the absence of corneal and pedal reflexes. The distal end of the arterial cannula was attached to a BP transducer for direct monitoring of BP.

Following a midline abdominal incision, the left lumbar postganglionic sympathetic nerve trunk was identified and dissected free of surrounding tissue. With the aid of an operating microscope the nerve was placed onto the bared tips of two Teflon-coated silver wire electrodes and the nerve–electrode junction insulated electrically from surrounding tissue with a sealant (Kwik-Cast Sealant, WPI, USA). The nerve activity was amplified using a low-noise differential amplifier (ENG Models 187B and 133, Baker Institute, VIC, Australia), filtered (bandpass 100–1000 Hz), rectified, and integrated at 0.5 s intervals. The signal was recorded using a MacLab data acquisition system (ADInstruments, NSW, Australia). The signal recorded at the end of the experiment after the injection of phenylephrine (5 μg/kg, iv) was deemed background noise. The LSNA was calculated by subtraction of background noise from the recorded nerve activity. The average integrated LSNA was calculated over a period of 1–2 min and expressed as a percentage of the resting period prior to the intracerebral administration of drugs.

Microinjections into the RVLM and Hypothalamus

For microinjections into the RVLM, each animal was placed prone and the head was mounted in a Stoelting stereotaxic frame such that both bregma and lambda were positioned on the same horizontal plane. Burr holes were drilled bilaterally into the occipital bone of the skull approximately 2 mm lateral of the mid-sagittal suture and 3.8 mm caudal of the lambdoid suture. The pressor region of the RVLM was identified functionally by microinjection of 50 nl of L-glutamate (0.1 M) which elicited a pressor response of at least 20 mmHg in arterial pressure (Kantzides et al., 2005). RVLM microinjections were made using the following coordinates: 3.7–4.0 mm caudal to lambdoid suture, 2 mm lateral to the midline, and 8.0 mm ventral to the surface of the dura.

For microinjections into the hypothalamic PVN, a midline reference point was marked 2 mm rostral to bregma. This was necessary because bregma was removed in some instances during the subsequent bone drilling procedure. Holes (approximately 4 mm in diameter) were drilled bilaterally into the skull centered 4.0 mm caudal from the reference point to allow microinjections of drugs into the PVN (stereotaxic coordinates: 3.8–4.1 mm caudal to the reference point, 0.5 mm lateral to midline, and 8.0 mm ventral to the surface of the dura).

All microinjections were made bilaterally using a fine glass micropipette (with a tip diameter of 50–70 μm). Micrinjection volumes were 100 nl/side and after each microinjection, the micropipette was left in place for approximately 1 min. To mark the injection sites, a small amount of rhodamine-tagged fluorescent microspheres was included in the microinjected solution (LumaFluor, NC, USA). The precise location of the microinjections was verified histologically at the end of each experiment.

EXPERIMENTAL PROTOCOL

In WKY rats bilateral microinjections were made into the RVLM (n = 16), PVN (n = 18), and into the area adjacent to the PVN (n = 8). Animals receiving microinjections into the RVLM were given vehicle (artificial CSF containing NaCl 124 mM,
KCl 3.0 mM, NaH₂PO₄·2H₂O 1.3 mM, MgCl₂·6H₂O 2.0 mM, NaHCO₃ 26 mM, glucose 10 mM, CaCl₂ 2.0 mM in Milli-Q water, buffered with carbogen), followed by either (i) five sequential doses of NaHS (0.2, 2, 20, 200, and 2000 pmol/side) or (ii) hydroxylamine (HA; 0.2, and 2 nmol/side, sequentially), and amino-oxyacetate (AOA; 0.1 and 1 nmol/side, sequentially) the order of HA and AOA was randomized. For microinjections into or out of the PVN the same protocol was followed except only three sequential doses of NaHS were administered (20, 200, and 2000 pmol/side). In three additional anesthetized rats, a bolus dose of NaHS (20 nmol) was administered into the lateral cerebral ventricle to determine the effects on the cardiovascular variables. In SHR rats, vehicle and NaHS (20–2000 pmol/side) were microinjected into the RVLM (n = 3) and PVN (n = 4) following a similar protocol. For all experiments, 10–15 min were allowed between each microinjection of drug. Mean arterial pressure (MAP), HR, and LSNA were monitored continuously. Resting levels prior to drug administration were recorded at 20 min before and immediately prior to the first intracerebral microinjection. At 1, 5, and 10 min after the administration of each dose of drug, MAP, HR, and LSNA were recorded for a duration of 1–2 min.

HISTOLOGY

At the end of each experiment, rats were killed using an overdose of pentobarbital sodium (325 mg/kg; Lethabarb, Virbac, NSW, Australia). The brain of each rat was then carefully removed and placed in a solution of 4% paraformaldehyde and 20% sucrose for 1 week. The medulla (for brains which had been microinjected into the RVLM) or the hypothalamus (for brains which had been microinjected into the PVN) were cut on a cryostat into 40 μm-thick sections and mounted onto gelatine subbed slides. The sections were then viewed wet under fluorescent microscopy to determine the position of the rhodamine beads which indicated the microinjection site. For the medulla, the caudal end of the facial nucleus, the nucleus ambiguous and the inferior olivary nuclei were identified in the wet sections, and the microinjection sites were mapped in relation to those structures. For the hypothalamus, after the center of the microinjections site was identified, the sections were dried before being stained with cresyl violet and cover-slipped with Depex mounting medium (BDH Lab Supplies, Poole, UK). Light microscopy was then used to re-examine the stained hypothalamic sections to determine the extent of the PVN and adjacent anatomical structures. The microinjection sites were subsequently mapped in relation to the PVN and the anatomical structures.

DETECTION OF H₂S-PRODUCING ENZYMES VIA SDS-PAGE AND WESTERN BLOTTING

Wistar kyoto rat brains were used for western blot analysis of CSE and CBS. The RVLM (n = 3) or hypothalamic PVN (n = 3) were punched out from frozen sections encompassing the entire rostral-caudal extent of each nucleus, using a blunted 20G needle. For the PVN the tissues from three animals were combined, as were those from the RVLM. The tissues were homogenized and suspended in sample buffer (sample buffer composition: 5% v/v Glycerine, 2.5% v/v mercaptoethanol, 1.5% SDS, 0.05 M TRIS/HCl pH 8, 0.05 mg/ml bromophenol blue). Samples were then heated to 65°C for 10 min. Protein concentration was determined from each sample and the samples were loaded onto 10% gels and separated by SDS-PAGE. After transfer to polyvinylidene difluoride membranes the blots were incubated with primary antibodies suspended in blocking buffer overnight [rabbit anti-CSE antibody (Proteintech Group Inc., USA) and mouse anti-CBS antibody (Abnova Corporation, Taiwan)]. The blots were then incubated with the appropriate secondary antibody (goat anti-rabbit, goat anti-mouse) conjugated to horseradish peroxidase for 1 h then developed by enhanced chemiluminescence (Millipore Kit). Dual color marker (Bio-Rad) was used for molecular weight determination. Recombinant protein of CSE and CBS (GST-tagged) were loaded on the gel to identify the band of interest (Abnova, Taiwan).

STATISTICS

The data from the in vivo studies were expressed as the change between the level immediately prior to each microinjection and the average of the level observed at 1 and 5 min after drug/vehicle administration. These time points corresponded to those used by others (Dave et al., 2008) and to the times at which an effect was most likely to be observed, as exemplified by the time course of effects following NaHS (data not shown). Since the vehicle was similar in each experiment, the vehicle responses were combined into a single control group for each brain region.

The average value of the changes was calculated and was subsequently compared between groups using one-way-ANOVA, followed by comparisons between the individual doses of drugs and control using Dunnett’s post hoc test for multiple comparisons. P < 0.05 (two-tailed) was considered statistically significant.

RESULTS

WKY RATS

CBS and CSE in RVLM and PVN

Figure 1A shows examples of the western blots used to determine the presence of CSE and CBS in the RVLM and PVN in the rat brain. The results show that the PVN and RVLM contain CBS. By contrast, in neither region was CSE detectable. As positive controls, we have previously shown that CSE is found in peripheral tissues such as the aorta and kidney (Al-Magableh and Hart, 2011).

Effect of NaHS microinjected into the RVLM

Sodium hydrogen sulfide (0.2–2000 pmol/side) microinjected into the RVLM resulted in small increases in MAP and HR but these were not significantly different from vehicle (Figure 1B). The LSNA responses were small and variable; LSNA decreased slightly following NaHS (0.2–20 pmol) and slightly increased or did not change following the higher doses, but was not significantly different from the vehicle response (Figure 1B).

Effect of HA and AOA microinjected into the rostral RVLM

The CBS inhibitors, AOA (0.1–1.0 nmol/side), and HA (0.2–2.0 nmol/side) microinjected into the RVLM did not significantly change any of the cardiovascular variables measured compared to vehicle (Figure 2). Following the highest dose of AOA, MAP, and HR tended to decrease but this was not seen with HA. Neither AOA nor HA significantly affected LSNA.
FIGURE 1 | (A) Western blot showing expression of cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) in punched out homogenates of the rostral ventrolateral medulla (RVLM) and hypothalamic paraventricular nucleus (PVN). The 63 kDa band corresponding to CBS protein was labeled in both PVN and RVLM samples. No CSE was observed in the PVN or RVLM. The GST-tagged CBS (86 kDa) and GST-tagged CSE (70 kDa) are shown. The native CSE protein is 44 kDa. (B) Changes in mean arterial pressure (MAP), heart rate (HR), and lumbar sympathetic nerve activity (LSNA) following vehicle (n = 13 for MAP and HR and n = 6 for LSNA) and amino-oxyacetate (AOA; 0.1–1.0 nmol/side) and hydroxylamine (HA; 0.2–2.0 nmol/side; n = 8 for MAP and HR; and n = 5 for LSNA) microinjected into the rostral ventrolateral medulla in WKY rats. AOA and HA are inhibitors of the enzyme cystathionine-β-synthase. Different shaded bars represent a different dose of drug as indicated.

Effect of NaHS microinjected into the PVN

Microinjection of NaHS (20–2000 pmol/side) into the PVN slightly increased MAP but this was not significantly different from the vehicle response (Figure 3). Similarly, the average changes in HR following NaHS were not significantly different from the vehicle response (Figure 3). LSNA was not markedly affected by microinjection of NaHS into the PVN (Figure 3). NaHS microinjected into the area surrounding the PVN also had no significant effect on MAP, HR, or LSNA (n = 4; data not shown).

Effect of HA and AOA microinjected into the PVN

Microinjection of AOA (0.1–1.0 nmol/side) into the PVN produced no significant change in MAP, HR, or LSNA compared to vehicle (Figure 4). Microinjection of HA (0.2 nmol/side) into the PVN resulted in a small but significant decrease in MAP and HR compared to vehicle (Figure 4). Microinjection of the higher dose of HA (2.0 nmol/side) into the PVN, however, did not elicit any significant effect on MAP and HR compared to vehicle (Figure 4). Neither dose of HA had any significant effect on LSNA compared to vehicle. Additionally, AOA and HA microinjected into the area surrounding the PVN also had no significant effect on MAP, HR, or LSNA (n = 4; data not shown).

Intracerebroventricular administration of NaHS

When NaHS (20 nmol) was administered into the lateral cerebral ventricle there were no significant effects on MAP (average change = 0.1 ± 1.6 mmHg), HR (−5 ± 3 b/min), or LSNA (0.2 ± 8.4%).
FIGURE 3 | Changes in mean arterial pressure (MAP), heart rate (HR), and lumbar sympathetic nerve activity (LSNA) following vehicle \((n = 14\) for MAP and HR and \(n = 7\) for LSNA) and the \(\text{H}_2\text{S}\) donor, \(\text{NaHS}\) \((20–2000\text { pmol/side}; n = 6\) for MAP and HR and \(n = 5\) for LSNA) microinjected into the hypothalamic paraventricular nucleus in WKY rats. Different shaded bars represent a different dose of \(\text{NaHS}\) as indicated.

SHR Rats

The average resting MAP prior to the microinjections into the brain in the SHR rats was \(96.4 \pm 3.9\text { mmHg}\) \((n = 7)\) which was significantly greater than in the WKY rats \((81.3 \pm 2.0, n = 34; P < 0.001)\). HR, however, was not significantly different between SHR and WKY rats \((343 \pm 9 \text { vs } 332 \pm 6, P < 0.001)\).

Microinjection into the RVLM

When \(\text{NaHS}\) \((20–200\text { pmol/side})\) was microinjected into the RVLM of SHR rats, there was no significant effect on MAP, and a slight increase following the 2000 pmol dose. In no instance was there any statistically significant difference from vehicle \((n = 3; \text{Figure 5A})\). On average, a small tachycardia was observed following each dose of \(\text{NaHS}\) but this was not significantly different from the vehicle response. LSNA tended to increase with the lower doses and decreased with the highest dose of \(\text{NaHS}\) but there was no significant difference compared to vehicle (Figure 5A).

Microinjection into the PVN

Microinjections of \(\text{NaHS}\) into the PVN of SHR rats did not significantly affect MAP, HR, or LSNA \((n = 5; \text{Figure 5B})\).

DISCUSSION

In the present study we found that the enzyme CBS but not CSE was present in the pressor region of the RVLM and in the PVN, suggesting \(\text{H}_2\text{S}\) may be endogenously produced in these brain regions. Microinjection of the \(\text{H}_2\text{S}\) donor, \(\text{NaHS}\), directly into these regions, however, did not significantly alter MAP, HR, or LSNA in WKY and SHR rats. In WKY rats, inhibition of the production of \(\text{H}_2\text{S}\), using inhibitors of CBS, in those brain regions also had no marked or consistent effects on the cardiovascular
endogenous H$_2$S within the PVN does not play a major role in the regulation of BP, HR, or LSNA in the normotensive state. In SHR rats, as in the WKY rats, we could not find any evidence suggesting H$_2$S in the PVN contributed to the regulation of the MAP, HR, and LSNA in the hypertensive state. Given the negative findings in the WKY rats, we did not pursue further investigations with HA and AOA in the PVN of SHR rats.

The RVLM is a key brain region involved in generating tonic sympathetic outflow (Guyenet, 2006). In the RVLM, microinjection of NaHS did not cause significant changes in MAP, HR, or LSNA compared to control. This was observed in WKY as well as SHR rats. The present results suggest that H$_2$S in the RVLM is not a key player in cardiovascular regulation in normotensive or hypertensive conditions. In order to observe the effects of endogenous H$_2$S, two inhibitors of CBS were employed, HA and AOA (Johnston and Balcar, 1974; Vidrio and Medina, 2007). Since both inhibitors affect the association of CBS with its co-factor, pyridoxal-5'-phosphate (PLP), the effects could be attributable to inhibition of PLP-dependent enzymes other than CBS. However, neither inhibitor microinjected into the RVLM of WKY rats significantly affected MAP, HR, or LSNA. These results indicate that, although CBS is present in the RVLM, H$_2$S produced locally in the RVLM does not have a major influence on BP, HR, or LSNA. Since we did not see any evidence to suggest a role for endogenous H$_2$S in WKY rats nor for exogenous H$_2$S in WKY and SHR rats, we did not further investigate the effects of HA or AOA in the SHR rats.

In contrast to the present work, a study appearing immediately prior to submission of the present manuscript, reported that NaHS microinjected into the RVLM of anesthetized rats induced dose-dependent, and relatively large, reductions in MAP, HR, and renal SNA (Guo et al., 2011). HA elicited the opposite cardiovascular effects (Guo et al., 2011). The reasons for the different responses compared to the present study are not known. In the present study the rats breathed spontaneously and a dorsal approach was used to functionally identify the pressor region of the RVLM. In the work by Guo et al., 2011, a ventral approach and visual identification of the RVLM was used, and the rats were ventilated. Stretching the chest wall during ventilation is known to enhance the excitatory drive arising from the RVLM and this can alter the responses to drugs administered into the RVLM (Cox and Brody, 1988). Functional identification of the pressor region of the RVLM was important in the present study to indicate (i) the correct placement of the microinjection and (ii) the RVLM was functional under the present experimental conditions and cardiovascular responses were clearly obtainable. It is noteworthy that similar doses were used in the present work and in the study by (Guo et al., 2011). It has also been reported that tachyphylaxis to repeated doses of NaHS may occur, at least in urogenital preparations (Patacchini et al., 2004). We do not believe this can account for the lack of responses in the present study since we have administered a single large dose of NaHS intracebroventricularly (20 nmol) and found no significant effect on the cardiovascular variables. Additionally, NaHS is well known to induce vasorelaxation after sequential doses are administered (Al-Magableh and Hart, 2011; Hart, 2011). In two previous studies in which NaHS administration elicited significant changes in MAP and HR, the experiments were conducted...
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FIGURE 6 (A) Schematic illustration showing the center of the microinjection sites within the rostral ventrolateral medulla (RVLM). Microinjections were made bilaterally but only unilateral sites are shown. Filled circles represent microinjection sites of NaHS and unfilled circles represent microinjection sites of amino-oxyacetate and hydroxylamine in WKY rats, unfilled triangles represent microinjection sites of NaHS in SHR rats. Approximate level caudal to bregma is indicated. Abbreviations: ROB, raphe obscurus; ION, inferior olivary nucleus; Sp5, spinal trigeminal tract; NAmb, nucleus ambiguous; Sol, solitary nucleus. (B) Schematic illustration showing the center of the microinjection sites within the hypothalamic paraventricular nucleus (PVN). Microinjections were made bilaterally but only unilateral sites are shown. Filled circles represent microinjection sites of NaHS and unfilled circles represent microinjection sites of amino-oxyacetate and hydroxylamine in WKY rats, unfilled triangles represent microinjection sites of NaHS in SHR rats. Approximate levels caudal to bregma are indicated. Abbreviations: Fx, fornix; AHA, anterior hypothalamic area; 3V, third ventricle; OT, optic tract; VMH, ventromedial hypothalamus.

in conscious rats. The changes in BP and HR reported were small (Dawe et al., 2008; Ufnal et al., 2008). The present work was conducted in the presence of anesthesia which may dampen BP and heart rate responses. Given the small magnitude of the responses to NaHS in the conscious rats, however, it is reasonable to question the physiological significance of H2S in cardiovascular regulation.

Opening of K<sub>ATP</sub> channels is believed to contribute to the effects of H2S, including vasodilation and cardioprotection (Zhao et al., 2001; Bian et al., 2006). Opening K<sub>ATP</sub> channels could decrease cell firing as a result of hyperpolarization. Indeed, a reduced discharge rate in spontaneously firing units in the RVLM after administration of a K<sub>ATP</sub> channel opener, adenosine, has been reported but there was no effect on BP or HR (Chen and He, 1998). In the PVN, a recent in vitro study using hypothalamic slices showed that the spontaneous firing of PVN neurons with projections to the spinal cord was reduced by adenosine; an effect mediated by opening of K<sub>ATP</sub> channels (Li et al., 2010). The present findings suggest that if H2S opens K<sub>ATP</sub> channels in the RVLM or in the PVN, then K<sub>ATP</sub> channels in those brain regions have little influence in the regulation of MAP, HR, or LSNA.

Relatively high mRNA levels of the Kir6.2 subtype of the K<sub>ATP</sub> channel have been demonstrated in the PVN (Dunn-Meynell et al., 1998). These channels may couple metabolic activity with neuronal excitability (Ashford et al., 1990), and are involved in sensing glucose and in regulating glucose metabolism (Zhang et al., 2004). Thus, although our studies suggest H2S in the PVN has no major role in regulating BP, HR, and LSNA, H2S in the PVN may perform other functions, which may involve metabolic regulation and these require further investigation.

CONCLUSION

We have demonstrated for the first time the presence of the enzyme CBS in two important cardiovascular regulatory regions, the RVLM and PVN. By contrast CSE was not observed in those brain regions. This is consistent with the current view that of those two enzymes, CBS is the main enzyme in the brain involved in the production of H2S. Our work also demonstrated there was no significant effect on BP, HR, and LSNA upon administration of the H2S donor, NaHS, into the RVLM and PVN of WKY and SHR rats, or following inhibition of CBS in the RVLM and PVN in WKY rats. Thus, we suspect that H2S in those regions is not playing a critical role in the regulation of BP, HR, and LSNA, at least, in the short term.
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