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Hydrogen sulfide activates TRPA1 and releases 5-HT from epithelioid cells of the chicken thoracic aorta

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26 **Abstract**

27 Epithelioid cells in the chicken thoracic aorta are chemoreceptor cells that release 5-HT in
28 response to hypoxia. It is likely that these cells play a role in chemoreception similar to that of glomus
29 cells in the carotid bodies of mammals. Recently, H₂S was reported to be a key mediator of carotid
30 glomus cell responses to hypoxia.

31 The aim of the present study was to reveal the mechanism of action of H₂S on 5-HT outflow from
32 chemoreceptor cells in the chicken thoracic aorta. The 5-HT outflow induced by NaHS, an H₂S donor,
33 and Na₂S₃, a polysulfide, was measured by using a HPLC equipped with an electrochemical detector.
34 NaHS (0.3–3 mM) caused a concentration-dependent increase in 5-HT outflow, which was significantly
35 inhibited by the removal of extracellular Ca²⁺. 5-HT outflow induced by NaHS (0.3 mM) was also
36 significantly inhibited by voltage-dependent L- and N-type Ca²⁺ channel blockers and a selective TRPA1
37 channel blocker. Cinnamaldehyde, a TRPA1 agonist, mimicked the secretory response to H₂S. 5-HT
38 outflow induced by Na₂S₃ (10 μM) was also inhibited by the TRPA1 channel blocker. Furthermore, the
39 expression of TRPA1 was localized to 5-HT-containing chemoreceptor cells in the aortic wall. These
40 findings suggest that the activation of TRPA1 and voltage-dependent Ca²⁺ channels is involved in H₂S-
41 evoked 5-HT release from chemoreceptor cells in the chicken aorta.

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43 **Keywords:** chicken aorta, 5-HT outflow, hydrogen sulfide, TRPA1 channel, Ca²⁺ channel.

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51 **1. Introduction**

52 Epithelioid cells in the luminal wall of the chicken thoracic aorta contain 5-hydroxytryptamine (5-HT).
53 These cells aggregate into clusters to form a band of about 1 mm in width (Yamamoto et al., 1989;
54 Miyoshi et al., 1995). Our laboratory has previously reported that these epithelioid cells are
55 chemoreceptor cells that evoke hypoxia-sensitive K^+ currents and 5-HT release (Ito et al., 1997; 1999). In
56 addition, these 5-HT-containing cells express neuronal nicotinic acetylcholine receptors and voltage-
57 dependent Na^+ , K^+ , and Ca^{2+} channels (Ito et al., 2001), and have 5-HT uptake activity (Delgermurun et
58 al., 2016), suggesting that these cells have similar properties to glomus cells in the carotid body of
59 mammals.

60 Hydrogen sulfide (H_2S) has been long considered as a toxic gas. However, it is well known that H_2S is
61 produced in the mammalian cells and plays important roles in various biological functions (Kimura, 2011).
62 H_2S is synthesized by several enzymes such as cystathionine- β -synthase (CBS) and cystathionine- γ -lyase
63 (CSE) (Olson, 2015). Recently, it has been reported that endogenous H_2S is a key mediator of the hypoxic
64 response in a variety of O_2 -sensitive tissues (Olson et al., 2006; 2008; 2015; Olson and Whitfield, 2010;
65 Dombkowski et al., 2011). In rodent carotid glomus cells, hypoxia triggers the synthesis of H_2S by CSE
66 and then increases catecholamine release (Makarenko et al., 2012). CBS- and CSE-catalyzed H_2S is also
67 known to mediate the carotid response to hypoxia (Li et al., 2010; Simith and Yuan, 2012; Parabhakar,
68 2012). However, the effect of H_2S on catecholamine release from glomus cells of the mammalian carotid
69 body is still unclear.

70 H_2S affects a variety of ion channels and receptors (Tang et al., 2010), including transient receptor
71 potential (TRP) A1 and V1 channels and voltage-dependent Ca^{2+} channels. TRPA1 and TRPV1 channels
72 are members of the TRP channel family, which serve to increase Ca^{2+} permeability in both excitable and
73 non-excitable cells (Venkatachalam and Montell, 2007; Gees et al., 2010). H_2S activates TRPA1 and
74 TRPV1 in the non-vascular smooth muscles of the urinary bladder, airways, and gastrointestinal tract

75 (Trevisani et al., 2005; Streng et al., 2008; Tang et al., 2010). Our group has previously shown that H₂S
76 evokes Ca²⁺ signals in rat sensory neurons (Myamoto et al., 2011), which induces 5-HT release from
77 RIN14B cells via the activation of TRPA1 (Ujike et al., 2015). In addition, mechanical hyperalgesia
78 (increased sensitivity to pain) and allodynia (producing pain by touch due to abnormal responses in the
79 neurons) induced by H₂S require activation of both TRPA1 and T-type Ca²⁺ channels in mice (Okubo et
80 al., 2012; Terada et al., 2015). Moreover, H₂S is oxidized to polysulfides (H₂S_n), which can activate ion
81 channels with a greater potency than that of H₂S. It has recently been reported that sodium trisulfide
82 (Na₂S₃) is found in the brain and activates astrocytes through stimulation of TRPA1, suggesting that it
83 acts as a signaling molecule in the brain (Kimura et al., 2013, 2015). Therefore, it seemed worthy to
84 examine which types of TRP channels are predominantly involved in the effect of H₂S and Na₂S₃ in
85 chemoreceptor cells.

86 In cultured smooth muscle cells from gastric fundus of mice, H₂S elicits in depolarization and then
87 activation of L-type Ca²⁺ channels, resulting in an increase in the intracellular Ca²⁺ level (Meng et al.,
88 2015). In carotid glomus cells, H₂S increases the intracellular Ca²⁺ concentration, which is inhibited by
89 the L-type Ca²⁺ channel blocker (Makarenko et al., 2012) and removal of extracellular Ca²⁺ (Peng et al.,
90 2010). Our previous study indicated that 5-HT-containing chemoreceptor cells in the chicken aorta have
91 voltage-dependent L- and N-type Ca²⁺ channels, and that 5-HT release following depolarization is
92 abolished by nifedipine and ω-conotoxin GVIA, L-, and N-type Ca²⁺ channel blockers, respectively (Ito et
93 al., 1999). Therefore, it is worth examining the involvement of voltage-dependent Ca²⁺ channels in H₂S-
94 induced 5-HT release from the chicken thoracic aorta.

95 In the present study, we investigated the effects of NaHS, an H₂S donor, and Na₂S₃ on 5-HT outflow
96 from the chicken thoracic aorta. To reveal the mechanism of action of H₂S on 5-HT outflow, we
97 examined the effects of TRP and voltage-dependent Ca²⁺ channel blockers, and detected the expression of
98 TRPA1 in 5-HT-containing epithelioid cells of the chicken thoracic aorta.

100 2. Materials and methods

101 2.1. Preparation of tissue samples

102 Male chickens (14–28 days after hatching) were deeply anesthetized by isoflurane and decapitated.
103 5-HT-containing cells aggregate in clusters in the inner wall of the chicken thoracic aorta as a
104 “chemoreceptive ring” (Miyoshi et al., 1995). A chicken aortic segment (about 5 mm in length) with 5-
105 HT-containing chemoreceptor cells was prepared as described previously (Delgermurun et al., 2016). All
106 experiments were performed under the regulations of the Institutional Animal Care and Use Committee of
107 the Graduate School of Veterinary Medicine, Hokkaido University. The animal facilities and animal care
108 programs were accredited by AAALAC international in the USA.

109

110 2.2. Measurement of 5-HT outflow

111 Hepes-buffered saline with the following composition was used (mM): NaCl 140, KCl 6, CaCl₂
112 2.5, MgCl₂ 1.2, Hepes 10, and glucose 10. The pH was buffered to 7.3 with NaOH. In Ca²⁺-free solution,
113 CaCl₂ was removed and 0.5 mM EGTA was added. The measurement of 5-HT outflow from an aortic
114 strip was carried out as described previously (Delgermurun et al., 2016). Briefly, the aortic strip was
115 placed in a sample tube containing Hepes-buffered saline solution (0.1 ml) with and without drugs and
116 then incubated at 37°C for 10 min. In our previous and preliminary studies, the 10-min incubation time
117 was enough to obtain measurable outflows of 5-HT and 5-HIAA, a 5-HT metabolite, in response to
118 various drugs (Ito et al., 1997; Delgermurun et al., 2016). Therefore, we set an incubation time for 10 min
119 to measure 5-HT and 5-HIAA. The secretory response of 5-HT was terminated by placing the tubes on ice.
120 After stopping the secretory response, the aortic tissue was transferred to another sample tube containing
121 0.4 N perchloric acid (0.2 ml) to extract 5-HT and 5-HIAA that remains in the tissue. To measure the
122 amounts of 5-HT and 5-HIAA released into the incubation medium, 4.4 N perchloric acid (10 µl) was

123 added to the medium to obtain a final concentration of 0.4 N. The sum of the amounts of 5-HT and 5-
124 HIAA in the tissue and incubation medium was regarded as the total amount of 5-HT. After centrifugation
125 of the sample tube containing tissue extract or incubation medium, K_2HPO_4 was added to the supernatant
126 to obtain a final concentration of 580 mM (pH 5–6). After centrifugation, the supernatant was subjected to
127 high-performance liquid chromatography (HPLC). The mobile phase was composed of the following
128 (mM): KH_2PO_4 - H_3PO_4 buffer (pH 3.5) 100, EDTA 0.04, sodium octasulfonic acid 1.16, and methanol
129 15–17%. The mobile phase was degassed using DG-350 (EICOM, Kyoto, Japan), at a flow rate of 0.5
130 ml/min. The samples were applied using an autosampler (System Instruments model 33, Tokyo, Japan) to
131 an ODS-column (EICOMPAK SC-50DS, 3.0×150 mm, EICOM), and 5-HT and 5-HIAA were detected
132 by an electrochemical detector (ECD-300, EICOM). 5-HT release (% of content) was expressed as a
133 percentage of total 5-HT content in the aortic strip.

134

135 2.3. Drugs

136 Following drugs were used. Sodium hydrosulfide (NaHS) was purchased from Strem Chemicals
137 (Newburyport, MA, USA). NaHS was used at concentrations from 0.03 mM to 3 mM because DRG
138 neurons were sensitivity to more than 1 mM and RIN14B cells were stimulated by 0.1 mM to 1 mM
139 (Miyamoto et al., 2011; Ujike et al., 2015). Na_2S_3 was purchased from Dojindo Molecular Technologies
140 Inc (Kumamoto, Japan). Nifedipine (1 μ M) (Wako, Osaka, Japan), ω -conotoxin GVIA (1 μ M) (Peptide
141 Institute, Osaka, Japan), ruthenium red (30 μ M) (Wako Pure Chemical Industries, Osaka, Japan),
142 HC030031 (0.3 mM), SB366791 (0.3 mM) (Bristol, UK), and trans-cinnamaldehyde (0.1-3 mM) (Aldrich
143 Chemistry, USA) were prepared from stock solutions and dissolved in Hepes-buffered saline solution.
144 Na_2S_3 (3-30 μ M)-containing aqueous solution was made just before each experiment.

145

146 2.4. Immunohistochemistry

147 The aortic strips containing the chemoreceptive ring were cut longitudinally, stretched into a sheet
148 and fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) at 4°C overnight. PBS with the
149 following composition was used (mM): NaCl 137, Na₂HPO₄ 8.1, KCl 2.7, KH₂PO₄ 1.5. For whole mount
150 immunohistochemical labeling, the tissues were treated with 1% Triton X-100 in PBS for 30 min three
151 times and incubated in blocking solution (1% normal horse serum with 1% Triton X-100 in PBS). The
152 tissues were incubated with 6% H₂O₂ in MeOH at room temperature for 1 h to block endogenous
153 peroxidase. The tissues were then incubated with primary antibody, anti-5-HT (1:50, 5-HT-H209, Novus
154 Biologicals, Littleton, CO, USA), and anti-TRPA1 antibody (1:1000, ab58844, Abcam, Cambridge, UK)
155 in blocking solution at 4°C for 2 days. Visualization of the primary antibodies was performed using
156 commercially available kits (VECTASTAIN Universal ABC kit Elite, Peroxidase substrate kit, Vector
157 laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. The tissues were
158 mounted on glass slides with glycerol and images were captured through an all-in-one fluorescence
159 microscope (BZ-710; Keyence, Osaka, Japan).

160 For double immunofluorescence, paraffin-embedded sections (3 µm) of aortic strips were
161 deparaffinized in xylene and rehydrated through a graded alcohol series. Tissues were treated with
162 methanol containing 0.3% H₂O₂. After blocking with 5% normal donkey serum, sections were incubated
163 with anti-5-HT (1:50, 5-HT-H209, Novus Biologicals) and anti-TRPA1 antibody (1:400, ab58844,
164 Abcam) at 4°C overnight. Next, the sections were treated with Alexa Fluor 546 conjugate donkey anti-
165 mouse IgG antibody (1:500, A10040, Thermo Fisher Scientific, Waltham, MA, USA) and Alexa Fluor
166 488 conjugate donkey anti-Rabbit IgG antibody (1:500, A-21206, Thermo Fisher Scientific) for 30 min at
167 room temperature for 5-HT and TRPA1, respectively. Nuclei were labeled by Hoechst 33342 (1:5000,
168 Dojindo, Kumamoto, Japan) for 5 min at room temperature. Digital images of sections were acquired
169 through an all-in-one fluorescence microscope (BZX-710; Keyence) equipping a 4',6-diamidino-2-
170 phenylindole filter (excitation 360/40; emission 460/50), green fluorescent protein filter (excitation

171 470/440; emission 525/50), and tetramethylrhodamine filter (excitation 545/25; emission 605/70) with a
172 metal halide lamp. For checking of the antibody specificity, we confirmed that negative control staining
173 without primary antibody incubation showed no positive reaction in immunofluorescence study.

174

175 2.5. Data analysis

176 All data were expressed as means $\pm$ S.E.M. Statistical comparisons between two groups were
177 performed by the unpaired Student's t-test. For multiple comparisons, one-way ANOVA, followed by
178 Dunnett' test or Williams' test, were used. A P value of less than 0.05 was considered significant.

179

180 3. Results

181 3.1. 5-HT outflow in response to H_2S

182 Chicken aortic tissues containing 5-HT-containing epithelioid cells were incubated at 37°C for 10 min
183 in Hepes-buffered solution containing various concentrations of NaHS (donor of H_2S). In the absence of
184 NaHS, 5-HT outflow was $6.0 \pm 0.5\%$ (n=14) during a 10 min incubation time. This outflow was
185 considered to be the basal level of 5-HT release from 5-HT-containing cells. NaHS caused a
186 concentration-dependent increase in 5-HT outflow (Fig. 1A), and NaHS (0.03-3 mM) caused a significant
187 release of 5-HT ($P<0.05$; Williams' test).

188 We have previously shown that 5-HT release from chemoreceptor cells in the chicken aorta during
189 hypoxia depends on Ca^{2+} influx through voltage-dependent L- and N-type Ca^{2+} channels (Ito et al., 1999).
190 Therefore, we examined the effect of extracellular Ca^{2+} on the 5-HT outflow induced by H_2S (Fig. 1B). 5-
191 HT outflow in the presence of 0.3-3 mM NaHS was statistically higher than the basal level in the absence
192 of NaHS ($P<0.05$; Williams' test). Although basal 5-HT outflow was not affected by the removal of
193 extracellular Ca^{2+} , 5-HT outflow in response to 0.3 mM NaHS was abolished by the removal of
194 extracellular Ca^{2+} ($P<0.01$; Student's t-test). On the other hand, NaHS-evoked 5-HT outflow in response

195 to 1 and 3 mM NaHS was significantly reduced ($P<0.01$; Student's t-test) but not abolished by the
196 removal of extracellular Ca^{2+} . These results suggest that 5-HT outflow induced by H_2S is largely
197 dependent on extracellular Ca^{2+} , whereas resting release of 5-HT is not. A small amount of 5-HT
198 appeared to be released even in the absence of extracellular Ca^{2+} in response to high concentrations of
199 H_2S .

200

201 *3.2. Contribution of voltage-dependent Ca^{2+} and TRP channels to 5-HT outflow induced by H_2S*

202 We investigated the effects of the voltage-dependent Ca^{2+} channel blockers on 5-HT outflow in
203 response to H_2S (Fig. 2A). 5-HT outflow induced by 0.3 mM NaHS was significantly higher than the
204 basal release ($P<0.01$; Dunnett's test) and was significantly inhibited by nifedipine (1 μM) or ω -conotoxin
205 GVIA (1 μM), which are L- and N-type voltage-dependent Ca^{2+} channel blockers, respectively ($P<0.01$;
206 Dunnett's test).

207 H_2S reportedly activates TRPV1 in the airway and the urinary bladder (Trevisani et al., 2005;
208 Patacchini et al., 2005). In addition, TRPA1 is activated by H_2S in neuronal and secretory cells
209 (Miyamoto et al., 2011; Ujike et al., 2015). Therefore, we investigated the effects of TRP channel
210 blockers on 5-HT outflow evoked by H_2S (Fig. 2B). 5-HT outflow induced by 0.3 mM NaHS was
211 significantly higher than the basal release ($P<0.01$; Dunnett's test) and was significantly inhibited by
212 ruthenium red (a non-selective TRP channel blocker) ($P<0.01$; Dunnett's test) and HC030031 (a selective
213 TRPA1 channel blocker) ($P<0.01$; Dunnett's test) but not by SB366791 (a selective TRPV1 channel
214 blocker). These results suggest that both TRPA1 and voltage-dependent L- and N-type Ca^{2+} channels are
215 involved in H_2S -evoked 5-HT release from the chemoreceptor cells in the chicken aorta.

216 TRPA1 is activated by a variety of plant-derived and environmental irritants, such as cinnamaldehyde,
217 isothiocyanate, allicin, and acrolein (Bandell et al., 2004; Bautista et al., 2006; Iwasaki et al., 2008), all of
218 which interact with cysteine residues in the ion channel protein (Bautista et al., 2006; Macpherson et al.,

219 2007). We then examined the effect of cinnamaldehyde, a TRPA1 agonist, on 5-HT outflow.
220 Cinnamaldehyde (0.1–3 mM) caused a concentration-dependent increase in 5-HT outflow (Fig. 3A), and
221 more than 0.3 mM of cinnamaldehyde caused a significant release of 5-HT ($P<0.05$; Williams' test).

222 Cinnamaldehyde (0.3 mM)-evoked 5-HT outflow was significantly higher than the basal release
223 ($P<0.01$; Dunnett's test) and was significantly inhibited by HC030031 ($P<0.01$; Dunnett's test) (Fig. 3B).
224 These results support our idea that TRPA1 is involved in H_2S -evoked 5-HT outflow from chemoreceptor
225 cells.

226 It has recently been reported that polysulfide such as Na_2S_3 excites mouse sensory neurons via the
227 activation of TRPA1 and causes acute pain (Hatakeyama et al., 2015). Therefore, we also examined the
228 effect of Na_2S_3 on 5-HT outflow from chemoreceptor cells. As shown in Fig 3C, Na_2S_3 (3–30 μM) caused
229 a concentration-dependent and significant increase in 5-HT outflow ($P<0.05$; Williams' test). Na_2S_3 (10
230 μM)-induced 5-HT outflow was significantly inhibited by HC030031 ($P<0.01$; Dunnett's test) (Fig 3D).
231 These results suggest that Na_2S_3 has a similar effect to H_2S to stimulate the chemoreceptor cells through
232 TRPA1.

233

234 3.3. Expression of TRPA1 in 5-HT-containing cells

235 5-HT-containing epithelioid cells exist in the wall of the chicken thoracic aorta (Miyoshi et al., 1995).
236 We examined whether or not TRPA1 protein is expressed in these 5-HT-containing chemoreceptor cells
237 by immunohistochemical analysis. TRPA1 immunoreactivity was detected in the chicken aorta (Fig. 4A
238 and B), and the area of TRPA1 expression was similar to that of 5-HT immunoreactive cells (i.e.,
239 chemoreceptive ring) (Fig. 4C and D). TRPA1 expression was localized to 5-HT-containing
240 chemoreceptor cells (Fig. 4G and J).

241

242 4. Discussion

243 In the present study, we demonstrated that H₂S and Na₂S₃ caused concentration-dependent increases
244 in 5-HT outflow from the chicken aorta comprising 5-HT-containing epithelioid cells, and that 5-HT
245 outflow in response to H₂S was sensitive to extracellular Ca²⁺ removal, TRPA1 blockers, and voltage-
246 dependent Ca²⁺ channel blockers. In addition, 5-HT outflow induced by Na₂S₃ was also sensitive to
247 TRPA1 channel blocker.

248 It has been previously reported that 5-HT-containing epithelioid cells in the chicken aorta are
249 chemoreceptor cells that release 5-HT in response to various stimuli such as nicotinic agonists,
250 depolarization, and hypoxia (Ito et al., 1997; 1999). In this study, we found that H₂S triggers 5-HT release
251 from chemoreceptor cells in the aortic tissues, and that this release was markedly reduced by the
252 removal of extracellular Ca²⁺, suggesting that H₂S causes Ca²⁺-dependent exocytosis of 5-HT. H₂S also
253 releases catecholamine from rat and trout adrenal chromaffin cells and 5-HT from RIN14B cells in a
254 Ca²⁺-dependent manner (Perry et al., 2009; Zhu et al., 2012; Ujike et al., 2015). On the other hand,
255 perceptible release of 5-HT in response to H₂S at high concentrations was observed even in the absence of
256 extracellular Ca²⁺. It is reported that some secretagogues cause catecholamine secretion from adrenal
257 chromaffin cells by mediating intracellular Ca²⁺ mobilization even in the absence of extracellular Ca²⁺
258 (Asano et al., 1995). In this experiment, although we did not examine this phenomenon precisely, similar
259 mechanisms might be involved in 5-HT outflow from chicken chemoreceptor cells in response to H₂S at
260 high concentrations.

261 TRPA1 channels are non-selective cationic channels permeable to Na⁺ and Ca²⁺ (Gees et al., 2010). It
262 has been reported that H₂S evokes time- and concentration-dependent increases in intracellular Ca²⁺
263 concentration ([Ca²⁺]_i) in CHO cells expressing mouse or human TRPA1 (Streng et al., 2008). In this
264 study, 5-HT outflow induced by H₂S was significantly inhibited by a non-selective TRP blocker
265 (ruthenium red) and a selective TRPA1 blocker (HC030031), and a TRPA1 agonist mimicked the
266 secretory response to H₂S. TRPA1 is expressed in many kinds of neuronal and non-neuronal cells and

tissues (Smith et al., 2004; Du et al., 2007; Atoyan et al., 2009; Lee et al., 2012; Cho et al., 2014). In this study, we also found that the TRPA1 is localized to 5-HT-containing chemoreceptor cells in the chicken aorta. It is also reported that H₂S is capable of activating TRPV1 in sensory neurons (Trevisani et al., 2005; Patacchini et al., 2005). However, TRPV1 is unlikely to be involved in 5-HT outflow induced by H₂S in chicken aortic chemoreceptor cells because 5-HT outflow was not inhibited by a selective TRPV1 blocker (SB366791). Our group has previously shown that H₂S stimulates rat sensory neurons and RIN14B cells via the activation of TRPA1 (Miyamoto et al., 2011; Ujike et al., 2015). In addition, it has recently been reported that polysulfide salts, i.e., sodium tri- and tetrasulfide (Na₂S₃ and Na₂S₄), activate TRPA1 channels in astrocytes much more potently than H₂S (Kimura et al., 2013). This was also the case in the present study of chemoreceptor cells where Na₂S₃ was increased 5-HT outflow by stimulating to TRPA1. Taken together, we conclude that TRPA1 plays an essential role in H₂S and Na₂S₃-induced 5-HT release from chemoreceptor cells in the chicken aorta.

It is well known that TRPA1 activation produces depolarization through the increase in Na⁺ permeability in addition to Ca²⁺ (Raisinghani et al., 2011), which in turn elicits further Ca²⁺ entry through voltage-dependent Ca²⁺ channels. There are two possible pathways to explain Ca²⁺ entry into chemoreceptor cells in response to H₂S; one could involve TRPA1 channels with high Ca²⁺ permeability, while the other could involve voltage-dependent Ca²⁺ channels stimulated by TRPA1 activation. In the present study, nifedipine and ω -conotoxin GVIA (L- and N-type voltage-dependent Ca²⁺ channel blockers, respectively) inhibited 5-HT outflow induced by H₂S. It has been reported that H₂S increases [Ca²⁺]_i and induces Ca²⁺ waves in cultured astrocytes and hippocampal slices, which is blocked by various types of Ca²⁺ channel inhibitors including L- and N-types (Nagai et al., 2004). Thus, we suggest that Ca²⁺ entry via L- and N-type Ca²⁺ channels, but not direct Ca²⁺ entry via TRPA1 channels, plays a major role in the 5-HT release triggered by H₂S in chicken aortic chemoreceptor cells. Additionally, the increase in 5-HT release induced by H₂S was greatly inhibited by either L- or N-type Ca²⁺ channel blockers. It seems likely

291 that Ca^{2+} entry into cells through both L- and N-type Ca^{2+} channels is necessary for 5-HT release in
292 response to H_2S .

293 H_2S is endogenously synthesized in mammalian tissues from L-cysteine by cystathionine- β -synthase
294 (CBS), cystathionine- γ -lyase (CSE), and 3-mercaptopyruvate sulfurtransferase in conjunction with
295 cysteine aminotransferase (Kamoun, 2004; Kimura, 2011; Mikami et al., 2011). Endogenous H_2S
296 functions as an oxygen sensor in various tissues (Olson, 2015). Recently, H_2S generated by CSE and CBS
297 was shown to be a physiological mediator of carotid body responses to hypoxia (Li et al., 2010;
298 Makarenko et al., 2012; Parabhakar, 2012). Exogenous H_2S activates carotid body type 1 cells in rat, cat,
299 and rabbit (Buckler, 2012; Jiao et al., 2015). The chemoreceptor cells in the chick aorta have similar
300 morphological and functional characteristics to mammalian carotid chemoreceptor cells (Miyoshi et al.,
301 1995; Ito et al., 2001). TRPA1 plays a critical role in the O_2 sensing of vagal and sensory neurons
302 (Takahashi et al., 2011; Mori et al., 2016). A TRPA1 antagonist causes a dose-dependent attenuation of
303 the hypoxic ventilator response, indicating that TRPA1 contributes to the hypoxic chemoreflex in mice
304 (Pokorski et al., 2014). In addition, TRPA1 is a major oxidant sensor in sensory neurons that is activated
305 by hypochlorite and hydrogen peroxide (Bessac et al., 2008). Our results suggest that it is likely that
306 endogenous H_2S also contributes to O_2 sensing of chemoreceptor cells in the chicken aorta via TRPA1.
307 Further research will be necessary to reveal the role of endogenous H_2S and TRPA1 in chicken aortic
308 chemoreceptor cells.

309 In conclusion, the present study shows that H_2S triggers the release of 5-HT from the chicken aorta
310 containing chemoreceptor cells. We suggest that H_2S activates TRPA1 expressed in chemoreceptor cells,
311 resulting in their depolarization and Ca^{2+} entry through voltage-dependent Ca^{2+} channels.

312

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316

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Figure Legends

Figure 1. 5-HT outflow in response to H₂S. (A) Concentration-dependent increase in 5-HT outflow (% of content) in response to H₂S. Dashed line indicates the basal release of 5-HT. (B) NaHS-induced 5-HT outflow for 10 min in the presence (black columns) or absence (hatched columns) of extracellular Ca^{2+} (0.5 mM EGTA). The values in the black columns were taken from Fig. 1A. Data are mean $\pm$ S.E.M (n=5-14). * $P < 0.05$ vs. basal release (Williams' test), ** $P < 0.01$ vs NaHS /2.5 mM Ca^{2+} (Student's t-test).

Figure 2. Effect of Ca^{2+} channel blockers and TRP channel antagonists on 5-HT outflow induced by H₂S. (A) 5-HT outflow (% of content) evoked by NaHS in the absence (control) and presence of

457 nifedipine (Nif) and ω -conotoxin GVIA (ω -CTX), (B) ruthenium red (RR), HC030031 (HC), and
458 SB366791 (SB). Data are mean $\pm$ S.E.M. (n=5–10). **P<0.01 vs control (Dunnett's test).

459

460 **Figure 3. Effect of cinnamaldehyde and Na₂S₃ on 5-HT outflow.** (A and C) Concentration-dependent
461 effect of cinnamaldehyde and Na₂S₃ on 5-HT outflow (% of content). Dashed lines indicate the basal
462 release of 5-HT. (B and D) 5-HT outflow evoked by cinnamaldehyde and Na₂S₃ in the absence (control)
463 and presence of HC030031 (HC). Data are mean $\pm$ S.E.M. (n=4–10). * P<0.05 vs basal release (Williams'
464 test), **P<0.01 vs control (Dunnett's test).

465

466 **Figure 4. Immunohistochemical localization of TRPA1 and 5-HT in the chicken aorta.** Distribution
467 of TRPA1 (A) and 5-HT immunoreactive cells (C) in the inner wall of the chicken aorta. High
468 magnification images of TRPA1 (B) and 5-HT (D) immunoreactive clusters from the boxed regions of
469 (A) and (C). Immunofluorescence labeling of TRPA1 (E) and 5-HT (F) in the chicken aorta. (G) TRPA1-
470 expressing 5-HT-containing chemoreceptor cells are indicated by arrows. (H, I and J) High magnification
471 images from boxed regions of (E), (F), and (G), respectively. Dotted lines are indicating the border
472 between vascular endothelium and lumen on the images. Scale bars indicate 400 μ m (A and C), 100 μ m
473 (B and D), 200 μ m (E-G), and 50 μ m (H-J).

Figure 1

Figure 2

Figure 3

Figure 4