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

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**Running title:** 5-HT release from epithelioid cells of chicken thoracic aorta

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Abstract

Epithelioid cells in the chicken thoracic aorta are chemoreceptor cells that release 5-HT in response to hypoxia. It is likely that these cells play a role in chemoreception similar to that of glomus cells in the carotid bodies of mammals. Recently, H$_2$S was reported to be a key mediator of carotid glomus cell responses to hypoxia.

The aim of the present study was to reveal the mechanism of action of H$_2$S on 5-HT outflow from chemoreceptor cells in the chicken thoracic aorta. The 5-HT outflow induced by NaHS, an H$_2$S donor, and Na$_2$S$_3$, a polysulfide, was measured by using a HPLC equipped with an electrochemical detector. NaHS (0.3–3 mM) caused a concentration-dependent increase in 5-HT outflow, which was significantly inhibited by the removal of extracellular Ca$^{2+}$. 5-HT outflow induced by NaHS (0.3 mM) was also significantly inhibited by voltage-dependent L- and N-type Ca$^{2+}$ channel blockers and a selective TRPA1 channel blocker. Cinnamaldehyde, a TRPA1 agonist, mimicked the secretory response to H$_2$S. 5-HT outflow induced by Na$_2$S$_3$ (10 µM) was also inhibited by the TRPA1 channel blocker. Furthermore, the expression of TRPA1 was localized to 5-HT-containing chemoreceptor cells in the aortic wall. These findings suggest that the activation of TRPA1 and voltage-dependent Ca$^{2+}$ channels is involved in H$_2$S-evoked 5-HT release from chemoreceptor cells in the chicken aorta.

Keywords: chicken aorta, 5-HT outflow, hydrogen sulfide, TRPA1 channel, Ca$^{2+}$ channel.
1. Introduction

Epithelioid cells in the luminal wall of the chicken thoracic aorta contain 5-hydroxytryptamine (5-HT). These cells aggregate into clusters to form a band of about 1 mm in width (Yamamoto et al., 1989; Miyoshi et al., 1995). Our laboratory has previously reported that these epithelioid cells are chemoreceptor cells that evoke hypoxia-sensitive K⁺ currents and 5-HT release (Ito et al., 1997; 1999). In addition, these 5-HT-containing cells express neuronal nicotinic acetylcholine receptors and voltage-dependent Na⁺, K⁺, and Ca²⁺ channels (Ito et al., 2001), and have 5-HT uptake activity (Delgermurun et al., 2016), suggesting that these cells have similar properties to glomus cells in the carotid body of mammals.

Hydrogen sulfide (H₂S) has been long considered as a toxic gas. However, it is well known that H₂S is produced in the mammalian cells and plays important roles in various biological functions (Kimura, 2011). H₂S is synthesized by several enzymes such as cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) (Olson, 2015). Recently, it has been reported that endogenous H₂S is a key mediator of the hypoxic response in a variety of O₂-sensitive tissues (Olson et al., 2006; 2008; 2015; Olson and Whitfield, 2010; Dombkowski et al., 2011). In rodent carotid glomus cells, hypoxia triggers the synthesis of H₂S by CSE and then increases catecholamine release (Makarenko et al., 2012). CBS- and CSE-catalyzed H₂S is also known to mediate the carotid response to hypoxia (Li et al., 2010; Simith and Yuan, 2012; Parabhakar, 2012). However, the effect of H₂S on catecholamine release from glomus cells of the mammalian carotid body is still unclear.

H₂S affects a variety of ion channels and receptors (Tang et al., 2010), including transient receptor potential (TRP) A1 and V1 channels and voltage-dependent Ca²⁺ channels. TRPA1 and TRPV1 channels are members of the TRP channel family, which serve to increase Ca²⁺ permeability in both excitable and non-excitable cells (Venkatachalam and Montell, 2007; Gees et al., 2010). H₂S activates TRPA1 and TRPV1 in the non-vascular smooth muscles of the urinary bladder, airways, and gastrointestinal tract.
(Trevisani et al., 2005; Streng et al., 2008; Tang et al., 2010). Our group has previously shown that H\textsubscript{2}S evokes Ca\textsuperscript{2+} signals in rat sensory neurons (Myamoto et al., 2011), which induces 5-HT release from RIN14B cells via the activation of TRPA1 (Ujike et al., 2015). In addition, mechanical hyperalgesia (increased sensitivity to pain) and allodynia (producing pain by touch due to abnormal responses in the neurons) induced by H\textsubscript{2}S require activation of both TRPA1 and T-type Ca\textsuperscript{2+} channels in mice (Okubo et al., 2012; Terada et al., 2015). Moreover, H\textsubscript{2}S is oxidized to polysulfides (H\textsubscript{2}Sn), which can activate ion channels with a greater potency than that of H\textsubscript{2}S. It has recently been reported that sodium trisulfide (Na\textsubscript{2}S\textsubscript{3}) is found in the brain and activates astrocytes through stimulation of TRPA1, suggesting that it acts as a signaling molecule in the brain (Kimura et al., 2013, 2015). Therefore, it seemed worthy to examine which types of TRP channels are predominantly involved in the effect of H\textsubscript{2}S and Na\textsubscript{2}S\textsubscript{3} in chemoreceptor cells.

In cultured smooth muscle cells from gastric fundus of mice, H\textsubscript{2}S elicits in depolarization and then activation of L-type Ca\textsuperscript{2+} channels, resulting in an increase in the intracellular Ca\textsuperscript{2+} level (Meng et al., 2015). In carotid glomus cells, H\textsubscript{2}S increases the intracellular Ca\textsuperscript{2+} concentration, which is inhibited by the L-type Ca\textsuperscript{2+} channel blocker (Makarenko et al., 2012) and removal of extracellular Ca\textsuperscript{2+} (Peng et al., 2010). Our previous study indicated that 5-HT-containing chemoreceptor cells in the chicken aorta have voltage-dependent L- and N-type Ca\textsuperscript{2+} channels, and that 5-HT release following depolarization is abolished by nifedipine and \textalpha-conotoxin GVIA, L-, and N-type Ca\textsuperscript{2+} channel blockers, respectively (Ito et al., 1999). Therefore, it is worth examining the involvement of voltage-dependent Ca\textsuperscript{2+} channels in H\textsubscript{2}S-induced 5-HT release from the chicken thoracic aorta.

In the present study, we investigated the effects of NaHS, an H\textsubscript{2}S donor, and Na\textsubscript{2}S\textsubscript{3} on 5-HT outflow from the chicken thoracic aorta. To reveal the mechanism of action of H\textsubscript{2}S on 5-HT outflow, we examined the effects of TRP and voltage-dependent Ca\textsuperscript{2+} channel blockers, and detected the expression of TRPA1 in 5-HT-containing epithelioid cells of the chicken thoracic aorta.
2. Materials and methods

2.1. Preparation of tissue samples

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

2.2. Measurement of 5-HT outflow

Hepes-buffered saline with the following composition was used (mM): NaCl 140, KCl 6, CaCl$_2$ 2.5, MgCl$_2$ 1.2, Hepes 10, and glucose 10. The pH was buffered to 7.3 with NaOH. In Ca$^{2+}$-free solution, CaCl$_2$ was removed and 0.5 mM EGTA was added. The measurement of 5-HT outflow from an aortic strip was carried out as described previously (Delgermurun et al., 2016). Briefly, the aortic strip was placed in a sample tube containing Hepes-buffered saline solution (0.1 ml) with and without drugs and then incubated at 37°C for 10 min. In our previous and preliminary studies, the 10-min incubation time was enough to obtain measurable outflows of 5-HT and 5-HIAA, a 5-HT metabolite, in response to various drugs (Ito et al., 1997; Delgermurun et al., 2016). Therefore, we set an incubation time for 10 min to measure 5-HT and 5-HIAA. The secretory response of 5-HT was terminated by placing the tubes on ice. After stopping the secretory response, the aortic tissue was transferred to another sample tube containing 0.4 N perchloric acid (0.2 ml) to extract 5-HT and 5-HIAA that remains in the tissue. To measure the amounts of 5-HT and 5-HIAA released into the incubation medium, 4.4 N perchloric acid (10 µl) was
added to the medium to obtain a final concentration of 0.4 N. The sum of the amounts of 5-HT and 5-HIAA in the tissue and incubation medium was regarded as the total amount of 5-HT. After centrifugation of the sample tube containing tissue extract or incubation medium, K$_2$HPO$_4$ was added to the supernatant to obtain a final concentration of 580 mM (pH 5–6). After centrifugation, the supernatant was subjected to high-performance liquid chromatography (HPLC). The mobile phase was composed of the following (mM): KH$_2$PO$_4$-H$_3$PO$_4$ buffer (pH 3.5) 100, EDTA 0.04, sodium octasulfonic acid 1.16, and methanol 15–17%. The mobile phase was degassed using DG-350 (EICOM, Kyoto, Japan), at a flow rate of 0.5 ml/min. The samples were applied using an autosampler (System Instruments model 33, Tokyo, Japan) to an ODS-column (EICOMPAK SC-50DS, 3.0 × 150 mm, EICOM), and 5-HT and 5-HIAA were detected by an electrochemical detector (ECD-300, EICOM). 5-HT release (% of content) was expressed as a percentage of total 5-HT content in the aortic strip.

2.3. Drugs

Following drugs were used. Sodium hydrosulfide (NaHS) was purchased from Strem Chemicals (Newburyport, MA, USA). NaHS was used at concentrations from 0.03 mM to 3 mM because DRG neurons were sensitivity to more than 1 mM and RIN14B cells were stimulated by 0.1 mM to 1 mM (Miyamoto et al., 2011; Ujike et al., 2015). Na$_2$S$_3$ was purchased from Dojindo Molecular Technologies Inc (Kumamoto, Japan). Nifedipine (1 μM) (Wako, Osaka, Japan), ω-conotoxin GVIA (1 μM) (Peptide Institute, Osaka, Japan), ruthenium red (30 μM) (Wako Pure Chemical Industries, Osaka, Japan), HC030031 (0.3 mM), SB366791 (0.3 mM) (Bristol, UK), and trans-cinnamaldehyde (0.1-3 mM) (Aldrich Chemistry, USA) were prepared from stock solutions and dissolved in Hepes-buffered saline solution. Na$_2$S$_3$ (3-30 μM)-containing aqueous solution was made just before each experiment.

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

For double immunofluorescence, paraffin-embedded sections (3 µm) of aortic strips were deparaffinized in xylene and rehydrated through a graded alcohol series. Tissues were treated with methanol containing 0.3% H₂O₂. After blocking with 5% normal donkey serum, sections were incubated with anti-5-HT (1:50, 5-HT-H209, Novus Biologicals) and anti-TRPA1 antibody (1:400, ab58844, Abcam) at 4°C overnight. Next, the sections were treated with Alexa Fluor 546 conjugate donkey anti-mouse IgG antibody (1:500, A10040, Thermo Fisher Scientific, Waltham, MA, USA) and Alexa Fluor 488 conjugate donkey anti-Rabbit IgG antibody (1:500, A-21206, Thermo Fisher Scientific) for 30 min at room temperature for 5-HT and TRPA1, respectively. Nuclei were labeled by Hoechst 33342 (1:5000, Dojindo, Kumamoto, Japan) for 5 min at room temperature. Digital images of sections were acquired through an all-in-one fluorescence microscope (BZX-710; Keyence) equipping a 4',6-diamidino-2-phenylindole filter (excitation 360/40; emission 460/50), green fluorescent protein filter (excitation
470/440; emission 525/50), and tetramethylrhodamine filter (excitation 545/25; emission 605/70) with a metal halide lamp. For checking of the antibody specificity, we confirmed that negative control staining without primary antibody incubation showed no positive reaction in immunofluorescence study.

2.5. Data analysis

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

3. Results

3.1. 5-HT outflow in response to \( H_2S \)

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

We have previously shown that 5-HT release from chemoreceptor cells in the chicken aorta during hypoxia depends on Ca\(^{2+}\) influx through voltage-dependent L- and N-type Ca\(^{2+}\) channels (Ito et al., 1999). Therefore, we examined the effect of extracellular Ca\(^{2+}\) on the 5-HT outflow induced by \( H_2S \) (Fig. 1B). 5-HT outflow in the presence of 0.3-3 mM NaHS was statistically higher than the basal level in the absence of NaHS (P<0.05; Williams’ test). Although basal 5-HT outflow was not affected by the removal of extracellular Ca\(^{2+}\), 5-HT outflow in response to 0.3 mM NaHS was abolished by the removal of extracellular Ca\(^{2+}\) (P<0.01; Student’s t-test). On the other hand, NaHS-evoked 5-HT outflow in response
to 1 and 3 mM NaHS was significantly reduced (P<0.01; Student’s t-test) but not abolished by the removal of extracellular Ca\(^{2+}\). These results suggest that 5-HT outflow induced by H\(_2\)S is largely dependent on extracellular Ca\(^{2+}\), whereas resting release of 5-HT is not. A small amount of 5-HT appeared to be released even in the absence of extracellular Ca\(^{2+}\) in response to high concentrations of H\(_2\)S.

3.2. Contribution of voltage-dependent Ca\(^{2+}\) and TRP channels to 5-HT outflow induced by H\(_2\)S

We investigated the effects of the voltage-dependent Ca\(^{2+}\) channel blockers on 5-HT outflow in response to H\(_2\)S (Fig. 2A). 5-HT outflow induced by 0.3 mM NaHS was significantly higher than the basal release (P<0.01; Dunnett’s test) and was significantly inhibited by nifedipine (1 µM) or ω-conotoxin GVIA (1 µM), which are L- and N-type voltage-dependent Ca\(^{2+}\) channel blockers, respectively (P<0.01; Dunnett’s test).

H\(_2\)S reportedly activates TRPV1 in the airway and the urinary bladder (Trevisani et al., 2005; Patacchini et al., 2005). In addition, TRPA1 is activated by H\(_2\)S in neuronal and secretory cells (Miyamoto et al., 2011; Ujike et al., 2015). Therefore, we investigated the effects of TRP channel blockers on 5-HT outflow evoked by H\(_2\)S (Fig. 2B). 5-HT outflow induced by 0.3 mM NaHS was significantly higher than the basal release (P<0.01; Dunnett’s test) and was significantly inhibited by ruthenium red (a non-selective TRP channel blocker) (P<0.01; Dunnett’s test) and HC030031 (a selective TRPA1 channel blocker) (P<0.01; Dunnett’s test) but not by SB366791 (a selective TRPV1 channel blocker). These results suggest that both TRPA1 and voltage-dependent L- and N-type Ca\(^{2+}\) channels are involved in H\(_2\)S-evoked 5-HT release from the chemoreceptor cells in the chicken aorta.

TRPA1 is activated by a variety of plant-derived and environmental irritants, such as cinnamaldehyde, isothiocyanate, allicin, and acrolein (Bandell et al., 2004; Bautista et al., 2006; Iwasaki et al., 2008), all of which interact with cysteine residues in the ion channel protein (Bautista et al., 2006; Macpherson et al.,
We then examined the effect of cinnamaldehyde, a TRPA1 agonist, on 5-HT outflow. Cinnamaldehyde (0.1–3 mM) caused a concentration-dependent increase in 5-HT outflow (Fig. 3A), and more than 0.3 mM of cinnamaldehyde caused a significant release of 5-HT ($P<0.05$; Williams’ test).

Cinnamaldehyde (0.3 mM)-evoked 5-HT outflow was significantly higher than the basal release ($P<0.01$; Dunnett’s test) and was significantly inhibited by HC030031 ($P<0.01$; Dunnett’s test) (Fig. 3B). These results support our idea that TRPA1 is involved in H$_2$S-evoked 5-HT outflow from chemoreceptor cells.

It has recently been reported that polysulfide such as Na$_2$S$_3$ excites mouse sensory neurons via the activation of TRPA1 and causes acute pain (Hatakeyama et al., 2015). Therefore, we also examined the effect of Na$_2$S$_3$ on 5-HT outflow from chemoreceptor cells. As shown in Fig 3C, Na$_2$S$_3$ (3-30 µM) caused a concentration-dependent and significant increase in 5-HT outflow ($P<0.05$; Williams’ test). Na$_2$S$_3$ (10 µM)-induced 5-HT outflow was significantly inhibited by HC030031 ($P<0.01$; Dunnett’s test) (Fig 3D). These results suggest that Na$_2$S$_3$ has a similar effect to H$_2$S to stimulate the chemoreceptor cells through TRPA1.

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

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

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

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

TRPA1 channels are non-selective cationic channels permeable to Na+ and Ca2+ (Gees et al., 2010). It has been reported that H2S evokes time- and concentration-dependent increases in intracellular Ca2+ concentration ([Ca2+]i) in CHO cells expressing mouse or human TRPA1 (Streng et al., 2008). In this study, 5-HT outflow induced by H2S was significantly inhibited by a non-selective TRP blocker (ruthenium red) and a selective TRPA1 blocker (HC030031), and a TRPA1 agonist mimicked the 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
that Ca$^{2+}$ entry into cells through both L- and N-type Ca$^{2+}$ channels is necessary for 5-HT release in response to H$_2$S.

H$_2$S is endogenously synthesized in mammalian tissues from L-cysteine by cystathionine-$\beta$-synthase (CBS), cystathionine-$\gamma$-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase in conjunction with cysteine aminotransferase (Kamoun, 2004; Kimura, 2011; Mikami et al., 2011). Endogenous H$_2$S functions as an oxygen sensor in various tissues (Olson, 2015). Recently, H$_2$S generated by CSE and CBS was shown to be a physiological mediator of carotid body responses to hypoxia (Li et al., 2010; Makarenko et al., 2012; Parabhakar, 2012). Exogenous H$_2$S activates carotid body type 1 cells in rat, cat, and rabbit (Buckler, 2012; Jiao et al., 2015). The chemoreceptor cells in the chick aorta have similar morphological and functional characteristics to mammalian carotid chemoreceptor cells (Miyoshi et al., 1995; Ito et al., 2001). TRPA1 plays a critical role in the O$_2$ sensing of vagal and sensory neurons (Takahashi et al., 2011; Mori et al., 2016). A TRPA1 antagonist causes a dose-dependent attenuation of the hypoxic ventilator response, indicating that TRPA1 contributes to the hypoxic chemoreflex in mice (Pokorski et al., 2014). In addition, TRPA1 is a major oxidant sensor in sensory neurons that is activated by hypochlorite and hydrogen peroxide (Bessac et al., 2008). Our results suggest that it is likely that endogenous H$_2$S also contributes to O$_2$ sensing of chemoreceptor cells in the chicken aorta via TRPA1. Further research will be necessary to reveal the role of endogenous H$_2$S and TRPA1 in chicken aortic chemoreceptor cells.

In conclusion, the present study shows that H$_2$S triggers the release of 5-HT from the chicken aorta containing chemoreceptor cells. We suggest that H$_2$S activates TRPA1 expressed in chemoreceptor cells, resulting in their depolarization and Ca$^{2+}$ entry through voltage-dependent Ca$^{2+}$ channels.

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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 Ca²⁺ (0.5 mM EGTA). The values in the black columns were taken from Fig. 1A. Data are mean ± S.E.M (n=5−14). *P<0.05 vs. basal release (Williams’ test), **P<0.01 vs NaHS /2.5 mM Ca²⁺ (Student’s t-test).

**Figure 2. Effect of Ca²⁺ 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...
nifedipine (Nif) and ω-conotoxin GVIA (ω-CTX), (B) ruthenium red (RR), HC030031 (HC), and SB366791 (SB). Data are mean ± S.E.M. (n=5–10). **P<0.01 vs control (Dunnett’s test).

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

Figure 4. Immunohistochemical localization of TRPA1 and 5-HT in the chicken aorta. Distribution of TRPA1 (A) and 5-HT immunoreactive cells (C) in the inner wall of the chicken aorta. High magnification images of TRPA1 (B) and 5-HT (D) immunoreactive clusters from the boxed regions of (A) and (C). Immunofluorescence labeling of TRPA1 (E) and 5-HT (F) in the chicken aorta. (G) TRPA1-expressing 5-HT-containing chemoreceptor cells are indicated by arrows. (H, I and J) High magnification images from boxed regions of (E), (F), and (G), respectively. Dotted lines are indicating the border between vascular endothelium and lumen on the images. Scale bars indicate 400 μm (A and C), 100 μm (B and D), 200 μm (E-G), and 50 μm (H-J).
Figure 1

A

Outflow of 5-HT (% of content)

NaHS (µM)

B

Outflow of 5-HT (% of content)

- 2.5 mM Ca²⁺
- Ca²⁺ removal (0.5 mM EGTA)

A

B

control NaHS (0.3 mM) NaHS (1 mM) NaHS (3 mM)
Figure 2

A

![Graph A](image)

<table>
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<th>ω-CTX (1 µM)</th>
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<td><strong>12</strong></td>
<td><strong>8</strong></td>
<td><strong>8</strong></td>
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</tbody>
</table>

B

![Graph B](image)

<table>
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<th>Basal Release</th>
<th>Control</th>
<th>RR (30 µM)</th>
<th>HC (0.3 mM)</th>
<th>SB (0.3 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0</strong></td>
<td><strong>8</strong></td>
<td><strong>8</strong></td>
<td><strong>8</strong></td>
<td><strong>8</strong></td>
</tr>
</tbody>
</table>
Figure 3

A

Outflow of 5-HT (% of content) vs. cinnamaldehyde (µM)

B

Outflow of 5-HT (% of content) for different treatments.

C

Outflow of 5-HT (% of content) vs. Na2S3 (µM)

D

Outflow of 5-HT (% of content) for different treatments.