HOKKAIDO UNIVERSITY

Title	Hydrogen sulfide activates TRPA1 and releases 5-HT from epithelioid cells of the chicken thoracic aorta
Author(s)	Delgermurun, Dugar; Yamaguchi, Soichiro; Ichii, Osamu et al.
Citation	Comparative Biochemistry and Physiology Part C : Toxicology & Pharmacology, 187, 43-49 https://doi.org/10.1016/j.cbpc.2016.05.004
Issue Date	2016-09
Doc URL	https://hdl.handle.net/2115/67099
Rights	©2016. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/
Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	Manuscript_Fig.1-4.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

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

Dugar Delgermurun[a], Soichiro Yamaguchi[a], Osamu Ichii[b], Yasuhiro Kon[b], Shigeo Ito[a], Ken-ichi Otsuguro[a]

[a] Laboratory of Pharmacology, [b] Laboratory of Anatomy, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

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

ms. has 24 pages, 4 figures.

*Corresponding author:
Dr. K. Otsuguro
Laboratory of Pharmacology, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University
Kita 18, Nishi 9
Sapporo 060-0818, Japan
Fax: +81-11-706-5220
E-mail: otsuguro@vetmed.hokudai.ac.jp

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_2S 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_2S on 5-HT outflow from chemoreceptor cells in the chicken thoracic aorta. The 5-HT outflow induced by NaHS, an H_2S donor, and Na_2S_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_2S. 5-HT outflow induced by Na_2S_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_2S-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-hydroxytriptamine (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 Ca^{2+} 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 (H_2S) has been long considered as a toxic gas. However, it is well known that H_2S is produced in the mammalian cells and plays important roles in various biological functions (Kimura, 2011). H_2S 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 H_2S is a key mediator of the hypoxic response in a variety of O_2-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 H_2S by CSE and then increases catecholamine release (Makarenko et al., 2012). CBS- and CSE-catalyzed H_2S is also known to mediate the carotid response to hypoxia (Li et al., 2010; Simith and Yuan, 2012; Parabhakar, 2012). However, the effect of H_2S on catecholamine release from glomus cells of the mammalian carotid body is still unclear.

H_2S affects a variety of ion channels and receptors (Tang et al., 2010), including transient receptor potential (TRP) A1 and V1 channels and voltage-dependent Ca^{2+} channels. TRPA1 and TRPV1 channels are members of the TRP channel family, which serve to increase Ca^{2+} permeability in both excitable and non-excitable cells (Venkatachalam and Montell, 2007; Gees et al., 2010). H_2S 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_2S evokes Ca^{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_2S require activation of both TRPA1 and T-type Ca^{2+} channels in mice (Okubo et al., 2012; Terada et al., 2015). Moreover, H_2S is oxidized to polysulfides (H_2S_n), which can activate ion channels with a greater potency than that of H_2S. It has recently been reported that sodium trisulfide (Na_2S_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_2S and Na_2S_3 in chemoreceptor cells.

In cultured smooth muscle cells from gastric fundus of mice, H_2S elicits in depolarization and then activation of L-type Ca^{2+} channels, resulting in an increase in the intracellular Ca^{2+} level (Meng et al., 2015). In carotid glomus cells, H_2S increases the intracellular Ca^{2+} concentration, which is inhibited by the L-type Ca^{2+} channel blocker (Makarenko et al., 2012) and removal of extracellular Ca^{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^{2+} channels, and that 5-HT release following depolarization is abolished by nifedipine and ω-conotoxin GVIA, L-, and N-type Ca^{2+} channel blockers, respectively (Ito et al., 1999). Therefore, it is worth examining the involvement of voltage-dependent Ca^{2+} channels in H_2S-induced 5-HT release from the chicken thoracic aorta.

In the present study, we investigated the effects of NaHS, an H_2S donor, and Na_2S_3 on 5-HT outflow from the chicken thoracic aorta. To reveal the mechanism of action of H_2S on 5-HT outflow, we examined the effects of TRP and voltage-dependent Ca^{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_2HPO_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_2PO_4-H_3PO_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_2S_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_2S_3 (3-30 μM)-containing aqueous solution was made just before each experiment.

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, Na_2HPO_4 8.1, KCl 2.7, KH_2PO_4 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% H_2O_2 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% H_2O_2. 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

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_2S 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_2S.

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

We investigated the effects of the voltage-dependent Ca^{2+} channel blockers on 5-HT outflow in response to H_2S (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_2S 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_2S 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_2S (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_2S-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.,

2007). 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_2S-evoked 5-HT outflow from chemoreceptor cells.

It has recently been reported that polysulfide such as Na_2S_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_2S_3 on 5-HT outflow from chemoreceptor cells. As shown in Fig 3C, Na_2S_3 (3-30 μM) caused a concentration-dependent and significant increase in 5-HT outflow ($P<0.05$; Williams' test). Na_2S_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_2S_3 has a similar effect to H_2S 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 H_2S and Na_2S_3 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 H_2S was sensitive to extracellular Ca^{2+} removal, TRPA1 blockers, and voltage-dependent Ca^{2+} channel blockers**.** In addition, 5-HT outflow induced by Na_2S_3 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 H_2S triggers 5-HT release from chemoreceptor cells in the aortic tissues, and that this release was markedly reduced by the removalof extracellular Ca^{2+}, suggesting that H_2S causes Ca^{2+}-dependent exocytosis of 5-HT. H_2S also releases catecholamine from rat and trout adrenal chromaffin cells and 5-HT from RIN14B cells in a Ca^{2+}-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 H_2S at high concentrations was observed even in the absence of extracellular Ca^{2+}. It is reported that some secretagogues cause catecholamine secretion from adrenal chromaffin cells by mediating intracellular Ca^{2+} mobilization even in the absence of extracellular Ca^{2+} (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 H_2S at high concentrations.

TRPA1 channels are non-selective cationic channels permeable to Na^+ and Ca^{2+} (Gees et al., 2010). It has been reported that H_2S evokes time- and concentration-dependent increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in CHO cells expressing mouse or human TRPA1 (Streng et al., 2008). In this study, 5-HT outflow induced by H_2S 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 H_2S. TRPA1 is expressed in many kinds of neuronal and non-neuronal cells and

267 tissues (Smith et al., 2004; Du et al., 2007; Atoyan et al., 2009; Lee et al., 2012; Cho et al., 2014). In this
268 study, we also found that the TRPA1 is localized to 5-HT-containing chemoreceptor cells in the chicken
269 aorta. It is also reported that H_2S is capable of activating TRPV1 in sensory neurons (Trevisani et al.,
270 2005; Patacchini et al., 2005). However, TRPV1 is unlikely to be involved in 5-HT outflow induced by
271 H_2S in chicken aortic chemoreceptor cells because 5-HT outflow was not inhibited by a selective TRPV1
272 blocker (SB366791). Our group has previously shown that H_2S stimulates rat sensory neurons and
273 RIN14B cells via the activation of TRPA1 (Miyamoto et al., 2011; Ujike et al., 2015). In addition, it has
274 recently been reported that polysulfide salts, i.e., sodium tri-and tetrasulfide (Na_2S_3 and Na_2S_4), activate
275 TRPA1 channels in astrocytes much more potently than H_2S (Kimura et al., 2013). This was also the case
276 in the present study of chemoreceptor cells where Na_2S_3 was increased 5-HT outflow by stimulating to
277 TRPA1. Taken together, we conclude that TRPA1 plays an essential role in H_2S and Na_2S_3-induced 5-HT
278 release from chemoreceptor cells in the chicken aorta.

279 It is well known that TRPA1 activation produces depolarization through the increase in Na^+
280 permeability in addition to Ca^{2+} (Raisinghani et al., 2011), which in turn elicits further Ca^{2+} entry through
281 voltage-dependent Ca^{2+} channels. There are two possible pathways to explain Ca^{2+} entry into
282 chemoreceptor cells in response to H_2S; one could involve TRPA1 channels with high Ca^{2+} permeability,
283 while the other could involve voltage-dependent Ca^{2+} channels stimulated by TRPA1 activation. In the
284 present study, nifedipine and ω-conotoxin GVIA (L- and N-type voltage-dependent Ca^{2+} channel blockers,
285 respectively) inhibited 5-HT outflow induced by H_2S. It has been reported that H_2S increases $[Ca^{2+}]_i$ and
286 induces Ca^{2+} waves in cultured astrocytes and hippocampal slices, which is blocked by various types of
287 Ca^{2+} channel inhibitors including L- and N-types (Nagai et al., 2004). Thus, we suggest that Ca^{2+} entry
288 via L- and N-type Ca^{2+} channels, but not direct Ca^{2+} entry via TRPA1 channels, plays a major role in the
289 5-HT release triggered by H_2S in chicken aortic chemoreceptor cells. Additionally, the increase in 5-HT
290 release induced by H_2S was greatly inhibited by either L- or N-type Ca^{2+} 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_2S.

H_2S is endogenously synthesized in mammalian tissues from L-cysteine by cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase in conjunction with cysteine aminotransferase (Kamoun, 2004; Kimura, 2011; Mikami et al., 2011). Endogenous H_2S functions as an oxygen sensor in various tissues (Olson, 2015). Recently, H_2S 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_2S 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_2S 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_2S and TRPA1 in chicken aortic chemoreceptor cells.

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

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (no. 26450440 to K.O.)

References

Asano, T., Ohta, T., Otsuguro, K., Ito, S., Nakazato, Y., 1995. Muscarinic receptor subtypes mediating catecholamine secretion and increase in intracellular Ca^{2+} concentration in adrenal chromaffin cells of the guinea pig. J. Vet. Med. Sci. 57, 1067-1071.

Atoyan, R., Shander, D., Botchkareva, NV., 2009. Non-neuronal expression of transient receptor potential type A1 (TRPA1) in human skin. J. Invest. Dermatol. 129, 2312-2315.

Bandell, M., Story, G. M., Hwang, S.W., Viswanath, V., Eid, S.R., Petrus, M.J., Earley, T.J., Patapoutian, A., 2004. Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron 41, 849-857.

Bautista, D.M., Jordt, S., Nikai, T., Tsuruda, P.R., Read, A.J., Poblete, J., Yamoah, E. N., Basbaum, A. I., Julius, D., 2006. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. Cell 124, 1262-1282.

Bessac, B. F., Sivula, M., Von Hehn, C.A., Escalera, J., Cohn, L., Jordt, S., 2008. TRPA1 is a major oxidant sensor in murine airway sensory neurons. J. Clin. Invest. 118, 1899-1910.

Buckler, K.J., 2012. Effects of exogenous hydrogen sulphide on calcium signaling, background (TASK) K channel activity and mitochondrial function in chemoreceptor cells. Pflugers Arch. 463, 743-754.

Cho, HJ., Callaghan, B., Bron, R., Bravo, D.M., Furness, J.B., 2014. Identification of enteroendocrine cells that express TRPA1 channels in the mouse intestine. Cell. Tissue. Res. 356, 77-82.

Delgermurun,D., Ito, S., Ohta, T., Yamaguchi, S., Otsuguro, K., 2016. Endogenous 5-HT outflow from

chicken aorta by 5-HT uptake inhibitors and amphetamine derivatives. J. Vet. Med. Sci. 78, 71-76.

Dombkowski, R.A., Naylor, M.G., Shoemaker, E., Smith, M., DeLeon, E.R., Stoy, G.F., Gao, Y., Olson, K.R., 2011. Hydrogen sulfide (H_2S) and hypoxia inhibit salmonid gastrointestinal motility: evidence for H_2S as an oxygen sensor. J. Experi. Biol. 214, 4030-4040.

Du, S., Araki, I., Yoshiyama, M., Nomura, T., Takeda, M., 2007. Transient receptor potential channel A1 involved in sensory transduction or rat urinary bladder through C-fiber pathway. Urology 70, 826-831.

Gees, M., Colsoul, B., Nilius, B., 2010. The role of transient receptor potential cation channels in Ca^{2+} signaling. Cold. Spring. Harb. Respect. Biol. 2:a003962.

Hatakeyama, Y., Takahashi, K., Tominaga, M., Kimura, H., Ohta, T., 2015. Polysulfedes evokes acute pain through the activation of nociceptive TRPA in mouse sensory neurons. Mol. pain. 11, 24.

Ito, S., Ohta, T., Nakazato, Y., 1997. Release of 5-hydroxytryptamine by hypoxia from epithelioid cells of chcken thoracic aorta. Br. J. Pharmacol. 122, 799-801.

Ito, S., Ohta, T., Nakazato, Y., 1999. Characteristics of 5-HT containing chemoreceptor cells of the aortic body. J. Physiol. 515, 49–59.

Ito, S., Ohta, T., Kasai, Y., Yonekubo, K., Nakazato, Y., 2001. Heterogeneity of neuronal nicotinic acetylcholine receptors in 5-HT-contaning chemoreceptor cells of the chicken aorta. Br. J. Pharmacol. 132, 1934-3940.

Iwasaki, Y., Tanabe, M., Kobata, K., Watanabe, T., 2008. TRPA1 agonists-allyl isothiocyanate and cynnamaldehyde-induce adrenalin secretion. Biosci. Biotechnol. Biochem. 72, 2608-2614.

Jiao, Y., Li, Q., Sun, B., Zhang, G., Rong, W., 2015. Hydrogen sulfide activates the carotid chemoreceptors in cat, rabbit and rat ex vivo preparations. Respir. Physiol. Neurobiol. 208, 15-20.

Kamoun, P., 2004. Endogenous production of hydrogen sulfide in mammals. Amino acids 26, 243-254.

Kimura, H., 2011. Hydrogen sulfide: its production, release and functions. Amino acids. 41, 113-121.

Kimura, Y., Mikami, Y., Osumi, K., Tsugane, M., Oka, J., Kimura, H., 2013. Polysulfides are possible H_2S-derived signaling molecules in rat brain. FASEB J. 27, 2451-2457.

Kimura, Y., Toyofuku, Y., Koike, S., Shibuya, N., Nagahara, N., Lefer, D., Ogasawara, Y., Kimura, H., 2015. Identification of H_2S_3 and H_2S produced by 3-mercaptopyruvate sulfurtransferase in the brain. Sci. Rep. 5, 14744.

Lee, S.M., Cho, Y.S., Kim, T.H., Mu, J., Ahn, D.K., Noguchi, K., Bae, Y.C., 2012. An ultrastructural evidence for the expression of transient receptor potential ankyrin 1 (TRPA1) in astrocytes in the rat trigeminal caudal nucleus. J. Chem. Neuroanat. 45, 45-49.

Li, Q., Sun, B., Wang, X., Jin, Z., Zhou, Y., Dong, L., Jiang, L., Rong, W., 2010. A crucial role for hydrogen sulfide in oxygen sensing via modulating large conductance calcium-activated potassium channels. Antioxid. Redox. Signal. 12, 1179-1189.

Macpherson, L. J., Dubin, A. E., Evans, M. J., Marr, F., Schultz, P. G., Cravatt, B. F., Patapoutian, A., 2007. Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. Nature 541-545.

Makarenko, V.V., Nanduri, J., Raghuraman, G., Fox, A.P., Gadalla, M.M., Kumar, G.K., Snyder, S.H., Prabhakar, N.R., 2012. Endogenous H_2S is required for hypoxic sensing by carotid body glomus cells. Am. J. Physiol. Cell. Physiol. 303, C916-C923.

Meng, X., Huang, X., Zhang, Ch., Liu, D., Lu, H., Kim, Y., Xu, W., 2015. Hydrogen sulfide-induced enhancement of gastric fundus smooth muscle tone is mediated by voltage-dependent potassium and calcium channels in mice. World. J. Gastroenterol. 21 (16), 4840-4851.

Mikami, Y., Shibuya, N., Kimura, Y., Nagahara, N., Ogasawara, Y., Kimura, H., 2011. Thioredoxin and duhydrolipoic acid are required for 3-mercaptopyruvate sulfurtransferase to produce hydrogen sulfide. Biochem. J. 439, 479-485.

385 Miyamoto, R., Otsuguro, K., Ito, S., 2011. Time- and concentration-dependent activation of TRPA1 by
386 hydrogen sulfide in rat DRG neurons. Neurosci. Lett. 499, 137-142.
387 Miyoshi, M., Hashimoto, Y., Kon, Y., Sugimura, M., 1995. Distribution of epithelioid cells in the wall of
388 the chicken aorta and their functional role as chemoreceptors. Anat. Record 242, 302-309.
389 Mori, Y., Takahashi, N., Polat, O.K., Kurokawa, T., Takeda, N., Inoue, M., 2016. Redox-sensitive
390 transient receptor potential channels in oxygen sensing and adaptation. Pflugers Arch. 468, 85-97.
391 Nagai, Y., Tsugane, M., Oka, J., Kimura, H., 2004. Hydrogen sulfide induces calcium waves in astrocytes.
392 FASEB J. 18, 557-559.
393 Okubo, K., Matsumura, M., Kawaishi, Y., Aoki, Y., Matsunami, M., Okawa, Y., Sekiguchi, F., Kawabat,
394 A., 2012. Hydrogen sulfide-induced mechanical hyperalgesia and allodyna require activation of
395 both $Ca_v3.2$ and TRPA1 channels in mice. Br. J. Pharmacol. 166, 1738-1743.
396 Olson, K.R., Dombkowski, R.A., Russell, M.J., Doellman, M.M., Head, S.K., Whitfield, N.L., Madden,
397 J.A., 2006. Hydrogen sulfide as an oxygen sensor/transducer in vertebrate hypoxic
398 vasoconstriction and hypoxic vasodilation. J. Experi. Biol. 209, 4011-4023.
399 Olson, K.R., Healy, M.J., Quin, Z., Skovgaard, N., Vulesevic, B., Duff, D.W., Whitfield, N.L., Yang, G.,
400 Wang, R., Perry, S.F., 2008. Hydrogen sulfide as an oxygen sensor in trout gill chemoreceptors.
401 Am J. Physiol. Regul. Integr. Comp. Physiol. 295, R669-R680.
402 Olson, K. R., Whitfield, N., 2010. Hydrogen sulfide and oxygen sensing in the cardiovascular system.
403 Antioxid. Redox. Signal. 12, 1219-1234.
404 Olson, K.R., 2015. Hydrogen sulfide as an oxygen sensor. Antioxid. Redox. Signal. 22, 377-397.
405 Parabhakar, N.R., 2012. Carbon monoxide (CO) and hydrogen sulfide (H_2S) in hypoxic sensing by the
406 carotid body. Respir. Physiol. Neurobiol. 184, 165-169.
407 Patacchini, R., Santicioli, P., Giuliani, S., Maggi, C.A., 2005. Pharmacological ingestigation of hydrogen
408 sulfide (H_2S) contractile activity in rat detrusor muscle. Eur. J. Pharmacol. 509, 171-177.

409 Peng, Y., Nanduri, J., Raghuraman, G., Souvannakitti, D., Gadalla, M.M., Kumar, G.K., Snyder, S.H.,
410 Prabhakar, N.R, 2010. H_2S mediates O_2 sensing in the carotid body. Proc. Natl. Acad. Sci. USA.
411 23, 10719-10724.
412 Perry, S.F., McNeill, B., Elia, E., Nagpal, A., Vulesevic, B., 2009. Hydrogen sulfide stimulates
413 catecholamine secretion in rainbow trout. Am. J. Physiol. Integr. Comp. Physiol. 296, 133-140.
414 Pokorski, M., Takeda, K., Sato, Y., Okada, Y., 2014. The hypoxic ventilator response and TRPA1
415 antagonism in conccious mice. Acta. Physiol. 210, 928-938.
416 Raisinghani, M., Zhong, L., Jeffry, J.A., Bishnoi, M., Pabbidi, R.M., Pimentel, F., Cao, D., Evans, M.S.,
417 Premkumor, L.S., 2011. Activation characteristics of transient receptor potential ankyrin 1 and its
418 role in nociception. Am. J. Physiol. Cell. Physiol. 301, C587-C600.
419 Smith, K.A., Yuan, J.X., 2012. H_2S, a gasotransmitter for oxygen sensing in carotid body. Focus on
420 "Endogenous H_2S required for hypoxic sensing by carotid body glomus cells". Am. J. Physiol.
421 Cell. Physiol. 303, C911-C912.
422 Smith, M.P., Beachan, D., Ensor, E., Koltzenburg, M., 2004. Cold-sensitive, methanol-insensitive
423 neurons in the murine symphathetic nervous system. Neuroreport 15, 1399-1403.
424 Streng, T., Axelsson, H.E., Hedlund, P., Andersson, D.A., Jordt, S., Bevan, S., Andersson, K., Högestätt,
425 E.D., Zygmunt, P.M, 2008. Distribution and function of the hydrogen sulfide-sensitive TRPA1 ion
426 channel inrat urinary bladder. Eur. Urol. 53, 391-400.
427 Takahashi, N., Kuwaki, T., Kiyonaka, S., Numata, T., Kozai, D., Mizuno, Y., Yamamoto, S., Naito, S.,
428 Knevels, E., Carmeliet, P., Oga, T., Kaneko, S., Suga, S., Nokami, T., Yoshida, J., Mori, Y., 2011.
429 TRPA1 underlies a sensing mechanism for O_2. Nature Chemical. Biol. 7, 701-711.
430 Tang, G., Wu, L., Wang, R., 2010. The pathophysiological significance of sulphur-containing gases
431 interaction of hydrogen sulfide with ion channels. Clin. Exp. Pharmacol. Physiol. 37, 753- 763.
432 Terada, Y., Fujimura, M., Nishimura, S., Tsubota, M., Sekiguchi, F., Kawabata, A., 2015. Roles of $Ca_v3.2$

and TRPA1 channels targeted by hydrogen sulfide in pancreatic nociceptive pricessing in mice with or without acute pancreatitis. J. Neurosci. Res. 93, 361-369.

Trevisani, M., Patacchini, R., Nicoletti, P., Gatti, R., Gazzieri, D., Lissi, N., Zagli, G., Creminon, C., Geppetti, P., Harrison, S., 2005. Hydrogen sulfide causes vanilloid receptor 1-mediated neurogenic inflammation in the airways. Br. J. Pharmacol. 145, 1123-1131.

Ujike, A., Otsuguro, K., Miyamoto, R., Yamaguchi, S., Ito, S., 2015. Bidirectional effects of hydrogen sulfide via ATP-sensitive K^+ channels and transient receptor potential A1 channels in RIN14B cells. Eur. J. Pharmacol. 764, 463-470.

Venkatachalam, K., Montell, C., 2007. TRP channels. Annu. Rev. Biochem. 76, 387-417.

Yamamoto, M., Kondo, H., Nagatu, I., 1989. Immunohistochemical demonstration of tyrosine hydroxylase, serotonin and neuropeptide tyrosine in the epitheilioid cells within arterial walls and carotid bodies of chicks. J. Anat. 167, 137-146.

Zhu, D., Yu, X., Sun, J., Li, J., Ma, X., Yao, W., 2012. H2S induces catecholamine secretion in rat adrenal chromaffin cells. Toxicol. 302, 40-43.

Figure Legends

Figure 1. 5-HT outflow in response to H_2S. (A) Concentration-dependent increase in 5-HT outflow (% of content) in response to H_2S. 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 ± 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 H_2S. (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 ± S.E.M. (n=5−10). **P<0.01 vs control (Dunnett's test).
459
460 **Figure 3. Effect of cinnamaldehyde and Na_2S_3 on 5-HT outflow.** (A and C) Concentration-dependent
461 effect of cinnamaldehyde and Na_2S_3 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 Na_2S_3 in the absence (control)
463 and presence of HC030031 (HC). Data are mean ± 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 μm (A and C), 100 μm
473 (B and D), 200 μm (E-G), and 50 μm (H-J).

Figure 1

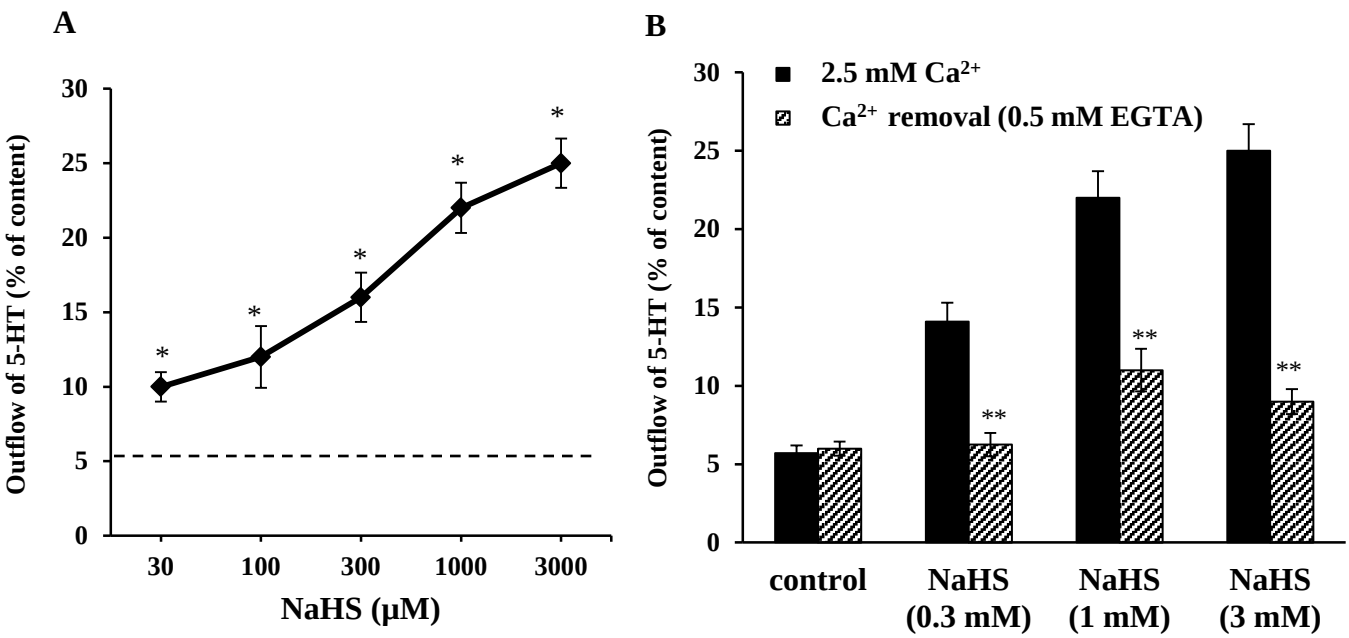

A
Outflow of 5-HT (% of content)
30
25
20
15
10
5
0
30
100
300
1000
3000
NaHS (μM)
B
2.5 mM Ca2+
Ca2+ removal (0.5 mM EGTA)
control
NaHS (0.3 mM)
NaHS (1 mM)
NaHS (3 mM)

Figure 2

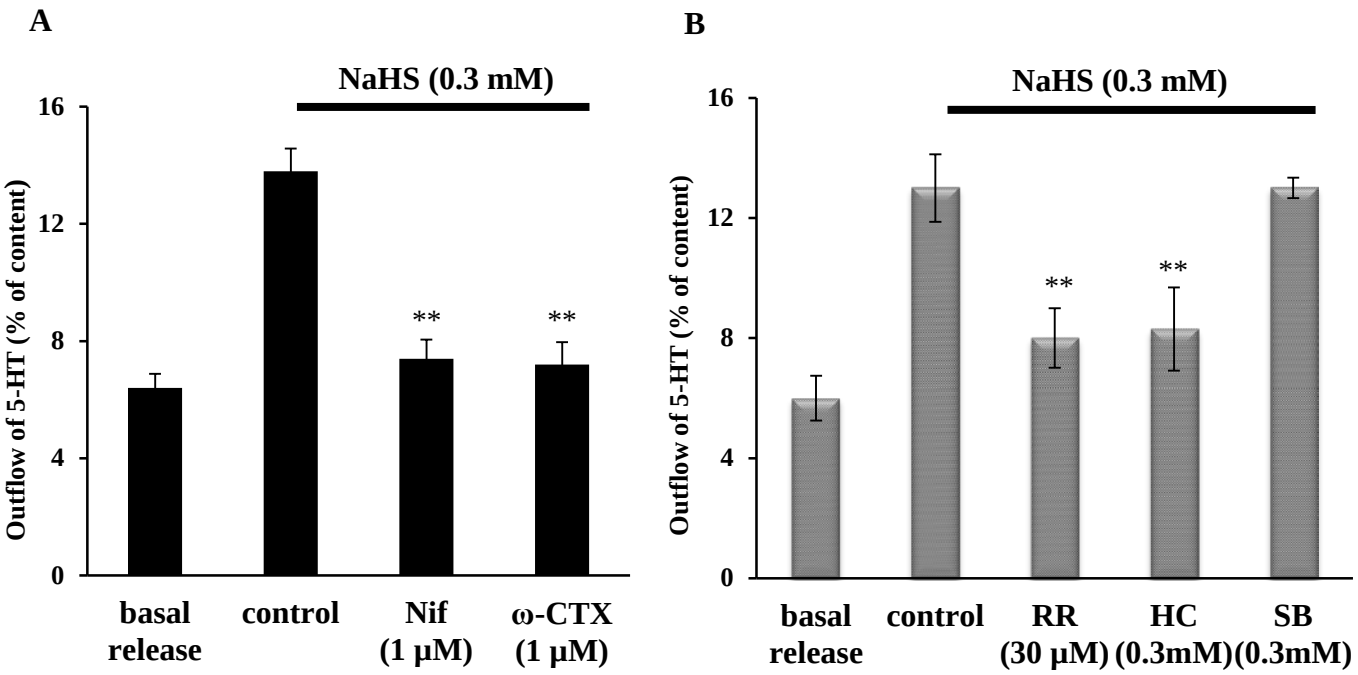

A
NaHS (0.3 mM)
Outflow of 5-HT (% of content)
16
12
8
4
0
**
**
basal release
control
Nif (1 μM)
ω-CTX (1 μM)
B
NaHS (0.3 mM)
Outflow of 5-HT (% of content)
16
12
8
4
0
**
**
basal release
control
RR (30 μM)
HC (0.3mM)
SB (0.3mM)

Figure 3

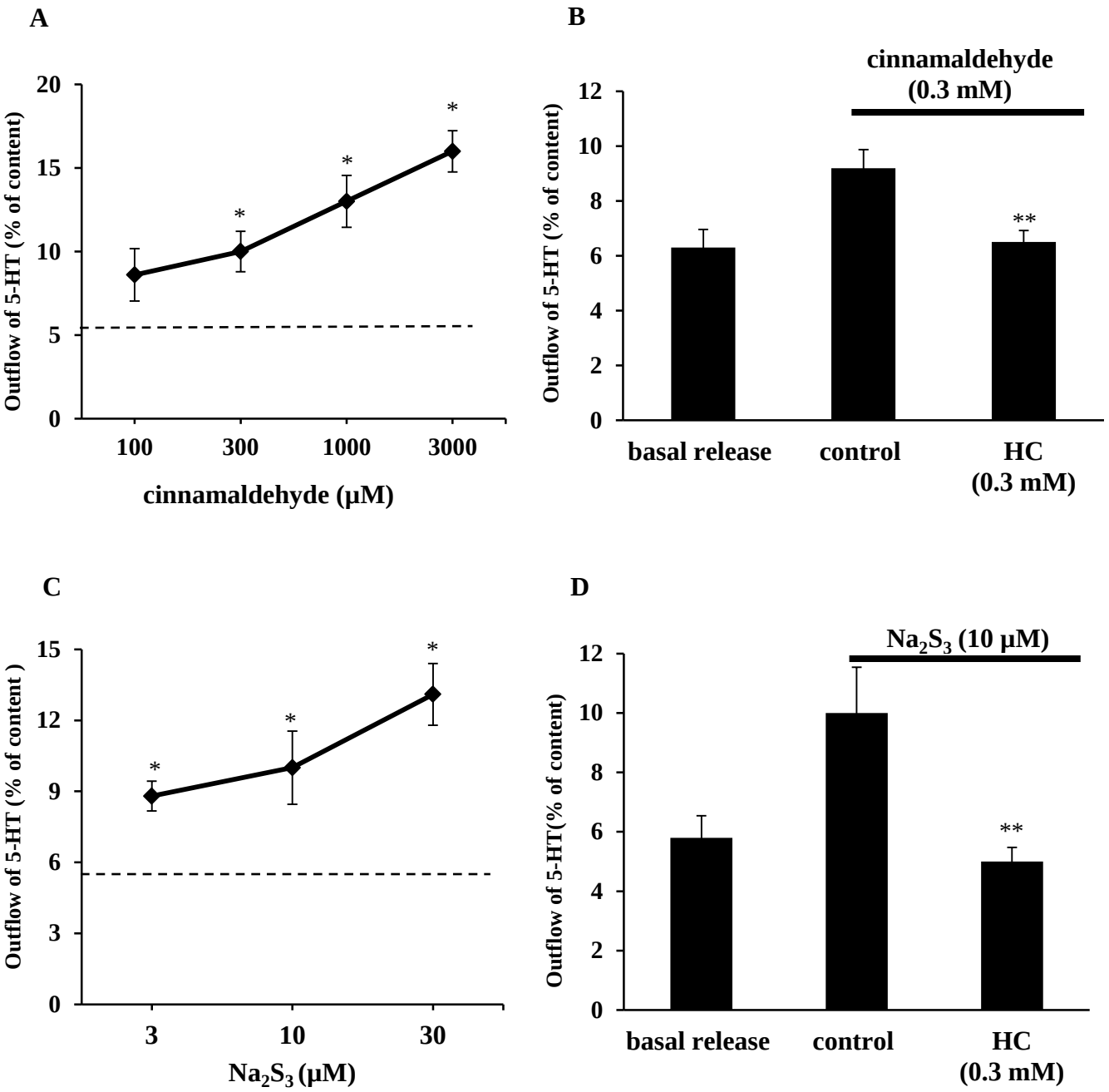
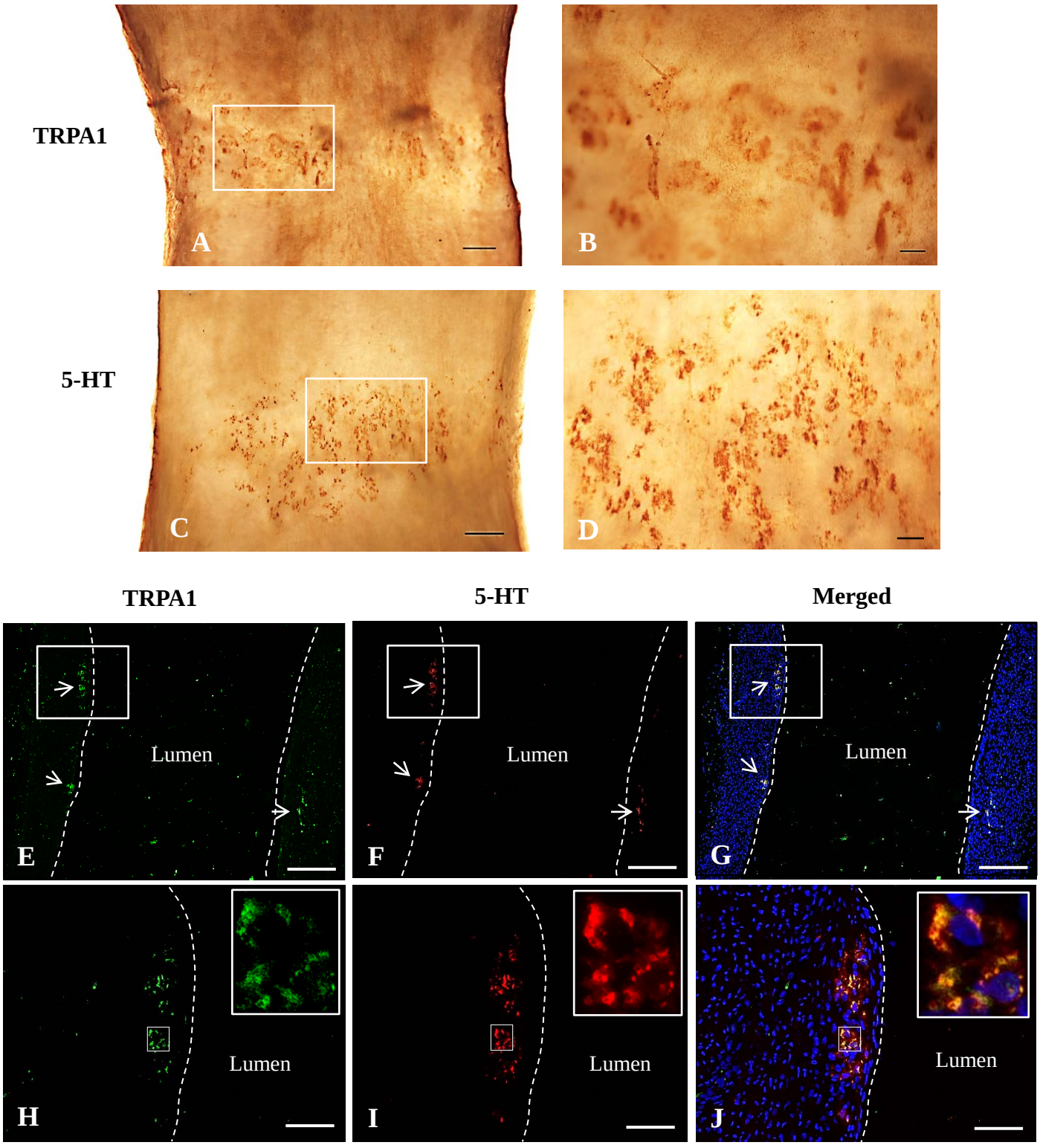

A
Outflow of 5-HT (% of content)
20
15
10
5
0
100
300
1000
3000
cinnamaldehyde (µM)
*
B
cinnamaldehyde
(0.3 mM)
Outflow of 5-HT (% of content)
12
10
8
6
4
2
0
**
basal release
control
HC
(0.3 mM)
C
Outflow of 5-HT (% of content)
15
12
9
6
3
0
3
10
30
Na2S3 (µM)
*
D
Na2S3 (10 µM)
Outflow of 5-HT(% of content)
12
10
8
6
4
2
0
**
basal release
control
HC
(0.3 mM)

Figure 4


TRPA1
5-HT
A
B
C
D
TRPA1
5-HT
Merged
Lumen
Lumen
Lumen
E
F
G
Lumen
Lumen
Lumen
H
I
J