Endogenous Hydrogen Sulfide Contributes to Tone Generation in Porcine Lower Esophageal Sphincter Via Na\(^+\)/Ca\(^{2+}\) Exchanger

Xiaopeng Bai,1 Eikichi Ihara,1 Katsuya Hirano,2 Yoshimasa Tanaka,1 Kayoko Nakano,3 Satomi Kita,3,5 Takahiro Iwamoto,4 Haruei Ogino,1 Mayumi Hirano,6 Yoshinao Oda,3 Kazuhiko Nakamura,1 and Yoshihiro Ogawa1,7

1Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; 2Department of Cardiovascular Physiology, Faculty of Medicine, Kagawa University, Kagawa Prefecture, Japan; 3Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; 4Department of Pharmacology, Faculty of Medicine, Fukuoka University, Fukuoka, Japan; 5Department of Pharmacology, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima, Japan; 6Division of Molecular Cardiology, Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; 7Department of Molecular and Cellular Metabolism, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan

SUMMARY

Endogenous hydrogen sulfide is continuously produced by 2 hydrogen sulfide-generating enzymes, cystathionine-β-synthase and 3-mercaptopyruvate sulfurtransferase, in porcine lower esophageal sphincter smooth muscle. Endogenous hydrogen sulfide contributes to lower esophageal sphincter myogenic tone generation by maintaining cytosolic Ca\(^{2+}\) concentration via the Na\(^+\)/Ca\(^{2+}\) exchanger.

BACKGROUND AND AIMS: Hydrogen sulfide (H\(_2\)S) is a major physiologic gastrotransmitter. Its role in the regulation of the lower esophageal sphincter (LES) function remains unknown. The present study addresses this question.

METHODS: Isometric contraction was monitored in circular smooth muscle strips of porcine LES. Changes in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) and force were simultaneously monitored in fura-2-loaded strips with front-surface fluorometry.

RESULTS: Porcine LES strips myogenically maintained a tetrodotoxin-resistant basal tone. Application of AOA (cystathionine-β-synthase inhibitor) or L-aspartic acid (L-Asp; 3-mercaptopyruvate sulfurtransferase inhibitor) but not DL-PAG (cystathionine-γ-lyase inhibitor), decreased this basal tone. The relaxant effects of AOA and L-Asp were additive. Maximum relaxation was obtained by combination of 1 mM AOA and 3 mM L-Asp. Immunohistochemical analyses revealed that cystathionine-β-synthase and 3-mercaptopyruvate sulfurtransferase, but not cystathionine-γ-lyase, were expressed in porcine LES. AOA+L-Asp–induced relaxation was accompanied by a decrease in [Ca\(^{2+}\)] and inversely correlated with the extracellular Na\(^+\) concentration ([Na\(^+\)]).
acid (AOA) has been shown to inhibit the tonic
was significantly augmented in the antral smooth muscle
sheets of Na⁺/Ca²⁺ exchanger transgenic mice compared
with wild-type mice.

CONCLUSIONS: Endogenous H₂S regulates the LES myogenic
tone by maintaining the basal [Ca²⁺], via Na⁺/Ca²⁺ exchanger.
H₂S-generating enzymes may be a potential therapeutic target
for esophageal motility disorders, such as achalasia. Understanding
the mechanisms controlling LES tone is crucial for gaining
insight into the treatment of these diseases.

Control of cytosolic Ca²⁺ concentration ([Ca²⁺]) and
Ca²⁺ sensitivity of the contractile apparatus play a key role
in myogenic regulation of the LES tone. The activation of the
L-type Ca²⁺ channel and Rho-associated kinase contribute to maintaining the myogenic tone by increasing
[Ca²⁺]i and Ca²⁺ sensitivity, respectively. The LES tone is also neurogenically regulated by excitatory and inhibitory
gastric pathways. The excitatory vagal pathway is mediated by cholinergic neurons, whereas the inhibitory
gastric pathway is mediated by the nonnoradrenergic/
noncholinergic neurons. The gasotransmitters nitric oxide and carbon monoxide act as neurotransmitters in the
inhibitory pathway. Hydrogen sulfide (H₂S) has been identified as a third gasotransmitter; however, its role
in the regulation of the LES tone remains unclear.

H₂S is synthesized endogenously by cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), or
3-mercaptopyruvate sulfurtransferase (3MST) in vertebrates. These 3 enzymes share a common substrate in
L-cysteine (L-Cys) for H₂S generation; however, they differ in the dependency on 2-pyridoxal-50-phosphate and
tissue distribution. CBS and CSE are 2-pyridoxal-50-phosphate-dependent, whereas 3MST is 2-pyridoxal-50-phosphate-independent. The expression of enzymes is regulated in a tissue-specific manner. H₂S carries out various physiologic functions in different tissues, including gastrointestinal smooth muscle. It opens ATP-sensitive K⁺ channels (KᵦTP channels) to control gastrointestinal contractility. H₂S also stimulates transient receptor potential vanilloid 1 channels in rat duodenum, causing duodenal smooth muscle contraction via the local release of substance P. The specific CBS inhibitor amino-oxyacetic acid (AOA) has been shown to inhibit the tonic
contraction in rat duodenum and mouse antrum, suggesting
the excitatory role of H₂S. However, the role of H₂S in the
regulation of LES contractility in either physiological or
pathologic situations remains largely unknown.

The present study examined the role of endogenous H₂S in the regulation of the basal tone of LES. NaHS, a
stable H₂S donor, has been used to examine the effects of exogenous H₂S, whereas inhibitors of H₂S-generating
enzymes have been used to investigate the functional role of endogenous H₂S. In the present study, the expression
of 3 major H₂S-generating enzymes in porcine LES was determined and the effects of H₂S-generating enzyme
inhibitors on the LES contractility were investigated.

Methods

All protocols for animal experiments were approved by the Animal Use and Care Committee of Fukuoka University and
Kyushu University. All authors had access to the study data and reviewed and approved the final manuscript.

Tissue Preparation of Porcine Lower Esophageal
Sphincter Circular Muscle Strips

A section of porcine esophagus containing the
esophagogastric junction was freshly obtained from a
local slaughterhouse and immediately transported to our
laboratory in normal extracellular solution containing 137.4 mM NaCl (137-NES). The specimen was cut open in the
longitudinal direction along the greater curvature of the stomach and pinned to the flat surface of a silicone rubber
plate with the mucosal side up. After removing the mucosal and submucosal layers, the circular smooth muscle
sheets of the LES were excised and cut into strips (5 × 2 mm) under a binocular microscope.

Na⁺/Ca²⁺ Exchanger Transgenic Mice

It has been shown that H₂S-generating enzyme CBS plays a role in myogenic tone generation in mouse antral smooth
muscle. Na⁺/Ca²⁺ exchanger (NCX) 1.3 transgenic (TG) mice were used to determine the association of endogenous
H₂S with NCX. NCX1.3 TG mice in a C57BL/6J background were generated as previously described. TG mice were

Abbreviations used in this paper: AOA, amino-oxyacetic acid; [Ca²⁺],
cystathionine-β-synthase; CBS, cystathionine-γ-lyase; CCh,
carbachol; CSE, cystathionine-γ-lyase; ES, extracellular solution; H₂S,
hydrogen sulfide; KᵦTP, channels; ATP-sensitive K⁺ channels; KES, K⁺
extracellular solution; L-Asp, L-aspartic acid; L-Cys, L-cysteine; LES,
lower esophageal sphincter; NAME, N⁴-nitro-L-arginine methyl ester;
[Na⁺]o, extracellular Na⁺ concentration; NCX, Na⁺/Ca²⁺ exchanger;
NES, normal extracellular solution; 3MST, 3-mercaptopyruvate sulfur-
transferase; PAG, propargylglycine; TEA, tetraethylammonium; TG,
transgenic; TTX, tetrodotoxin.

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housed in the facility of Fukuoka University, and wild-type mice were housed in Kyushu University, under 12-hour light/dark conditions and ambient temperature, with ad libitum access to water and food. Mice weighing 20–25 g (10–15 weeks, both male and female) were used in experiments. After the mice were sacrificed by cervical dislocation, the entire stomach was quickly excised and placed in ice-cold 137-NES. The stomach was cut open along the greater curvature and pinned to the base of a silicone dish, mucosal side up. The gastric antrum was cut along the circular axis. The mucosal and submucosal layers were carefully removed using fine forceps under a binocular microscope. Antral smooth muscle sheets (5 × 4 mm²) were then cut out and subjected to Fura-PE3 fluorometry.

**Force Measurement With Porcine Lower Esophageal Sphincter Circular Muscle Strips**

The porcine LES circular muscle strips were mounted vertically on a TB-612T force transducer (Nihon Koden, Tokyo, Japan) in an organ bath containing 5 mL 137-NES. The strips were then stretched to 1.3 times the resting length. Changes in isometric force were monitored at 37°C. During the equilibration period, strips were stimulated with 118 mM extracellular solution (118-KES) 4–5 times every 10 minutes. The extent of force development was expressed in % force, assigning the levels of force obtained at rest and at peak contraction induced by 118-KES as 0% and 100%, respectively, unless otherwise specified.

**Fura-PE3 Front-Surface Fluorometry With Porcine Lower Esophageal Sphincter Circular Muscle Strips and Mouse Antral Smooth Muscle Sheets**

Changes in [Ca²⁺], in porcine LES circular muscle strips and mouse antral smooth muscle sheets were monitored using fura-PE3 front-surface fluorometry. In brief, for fura-PE3 loading, the porcine LES strips were incubated in Dulbecco-modified Eagle medium containing 50 μM fura-PE3 in the form of acetoxyethyl ester (fura-PE3/AM), 250 mM probenecid, and 5% fetal bovine serum for 90 minutes at 37°C under aeration with 5% CO₂ and 95% O₂. ²¹,²² The mouse antral smooth muscle sheets were incubated in 137-NES containing 25 μM fura-PE3/AM and 1 μM probenecid for 60 minutes at 37°C in room air. The fura-PE3-loaded specimens were mounted vertically on a TB-612T force transducer in an organ bath containing 5 mL 137-NES and were stretched to 1.3 times their resting length. The specimens were stimulated with 118-KES 4–5 times every 10 minutes before starting the protocols. Changes in the fluorescence intensity of the fura-PE3-Ca²⁺ complex were monitored by a front-surface fluorimeter (CAM-OF3; Japan Spectroscopic Co, Tokyo, Japan), as previously described. ²³

The fluorescence intensities (500 nm) at 340 nm (F₃₄₀) and 380 nm (F₃₈₀) excitation and their ratio (F₃₄₀/F₃₈₀) were continuously monitored. ²² In porcine LES circular muscle strips, changes in [Ca²⁺], and force were simultaneously monitored. Carbachol (CCh) induced stable and reproducible responses in porcine LES circular muscle strips. Therefore, the levels of [Ca²⁺], and force obtained at rest and at peak contraction induced by 10 μM CCh were assigned values of 0% and 100%, respectively. In mouse antral smooth muscle sheets, changes in [Ca²⁺], induced by 50 μM ionomycin and subsequent incubation in Ca²⁺-free solution containing 0.5 mM ethyleneglycol-bis(2-aminoethyl)ether)-N,N,N’,N’-tetraacetic acid were recorded at the end of each experimental protocol. The level of [Ca²⁺], obtained at peak elevation induced by ionomycin and that obtained in the Ca²⁺-free solution were assigned values of 100% and 0%, respectively.

**Immunohistochemistry of Hydrogen Sulfide–Generating Enzymes in Porcine Lower Esophageal Sphincter**

A portion of LES of the porcine esophagus was fixed in 4% paraformaldehyde in phosphate-buffered saline for 24 hours and embedded in paraffin. The paraffin blocks were cut into 4-μm-thick sections. The sections were deparaffinized in xylene and rehydrated in ascending dilutions of ethanol. After blocking with 3% nonfat dry milk in phosphate-buffered saline, the samples were incubated with either anti-CBS, anti-MST, or anti-CSE antibodies (1:50 dilution) in phosphate-buffered saline containing 3% nonfat dry milk at 4°C overnight. For a negative control, the overnight incubation was performed without any primary antibodies. After a 40-minute incubation at room temperature with a horseradish peroxidase-conjugated secondary antibody, the sections were developed with 3,3’-diaminobenzidine, and counterstained with hematoxylin. Microscopic images were obtained with a ×40 objective lens (Nikon, Tokyo, Japan), and images were captured with a CCD camera (Nikon) and saved as JPEG files.

**Solutions and Drugs**

The composition of 137-NES was as follows (in mM): 137.4 NaCl, 5.9 KCl, 1.2 CaCl₂, 1.2 MgCl₂, 11.5 glucose, and 11.6 HEPES, pH 7.3. 118-KES (isotonic) was prepared by replacing NaCl with equimolar KCl in 137-NES. The composition of 118-KES was as follows (in mM): 25.3 NaCl, 118.0 KCl, 1.2 CaCl₂, 1.2 MgCl₂, 11.5 glucose, and 11.6 HEPES, pH 7.3. Hypertonic 118-KES was prepared by adding KCl to 137-NES to achieve a final concentration of 118 mM. Extracellular solutions (ES) of 25, 40, and 80 mM NaCl (25-ES, 40-ES, and 80-ES, respectively) were prepared by replacing NaCl with equimolar Tris-HCl (pH 7.3, adjusted by KOH) in 137-NES. Tetrodotoxin (TTX), tetraethylammonium (TEA), AOA, propargylglycine (PAG), L-aspartic acid (L-Asp), L-Cys, ionomycin calcium salt, strophanthidin, nifedipine, and fetal bovine serum were purchased from Sigma (St. Louis, MO). N⁵-nitro-L-arginine methyl ester (L-NAME) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Fura-PE3/AM was purchased from Texas Fluorescence Laboratory (Austin, TX). Anti-CBS monoclonal antibody (sc-67154) and anti-CSE polyclonal antibody (sc-135203) were purchased from Santa Cruz Biotechnology (Dallas, TX).
Anti-MST polyclonal antibody (HPA001240) was purchased from Atlas antibodies AB (Stockholm, Sweden).

**Data Analysis**

Each sample was obtained from a different animal, indicating that “n” means the number of animals used in the present study. All data are expressed as the mean ± the standard error of the mean. Student t test was used to determine statistical significance between 2 groups. An analysis of variance followed by Dunnett test was used to determine statistical significance among 3 or more groups. A value of $P < .05$ was considered significant.

**Results**

**Relaxant Effect of Inhibitors of Hydrogen Sulfide–Generating Enzymes in Porcine Lower Esophageal Sphincter Circular Smooth Muscle**

To evaluate the functional role of endogenous H$_2$S in the generation of basal tone of LES, we examined the effects of inhibitors of 3 H$_2$S-generating enzymes (CBS, 3MST, and CSE) on the resting level of tension (Figure 1). AOA, an inhibitor of CBS, and L-Asp, an inhibitor of 3MST, induced concentration-dependent relaxation (Figure 1A and B). The maximum relaxation obtained with 1 mM AOA and 3 mM

![Figure 1. The relaxant effect of inhibitors of H$_2$S-generating enzymes in porcine LES circular smooth muscle.](image)

(A, B) Representative recordings of the relaxant responses induced by AOA (A, n = 4) and L-Asp (B, n = 4). (C) Concentration-dependent responses to inhibitors of H$_2$S-generating enzymes (0.1–3 M) are summarized. (D–F) Representative recordings of restored tension by the addition of L-Cys in the presence of AOA (D, n = 4), L-Asp (E, n = 4), and both (F, n = 4). (G) Summary of the maximum relaxation induced by 1 mM AOA, 3 mM L-Asp, and 1 mM PAG in esophageal body (n = 4) and LES (n = 4). (H) Summary of the concentration-dependent responses to NaHS (n = 6). Force is expressed as %, with the levels of force obtained at rest in NES and at peak contraction induced by 118-KES set at 0% and 100%, respectively. Data are means ± SEM. **$P < .01$ versus the resting level (0%) or between indicated 2 groups.
L-Asp was \(-19.7\% \pm 3.10\%\) (n = 4) and \(-19.5\% \pm 3.47\%\) (n = 4) (Figure 1C). However, PAG, an inhibitor of CSE, induced no relaxation even at 3 mM (Figure 1C). The relaxant effects observed with AOA and L-Asp were partly reversed by the addition of L-Cys, which serves as a substrate for the generation of H\(_2\)S (Figure 1D and E). The extent of contraction induced by 10 mM L-Cys reached \(4.8\% \pm 1.38\%\) (n = 4) and \(12.0\% \pm 2.54\%\) (n = 4), in the presence of 1 mM AOA and 3 mM L-Asp, respectively. The combination of 1 mM AOA and 3 mM L-Asp induced a significantly larger relaxation (\(-27.4\% \pm 2.55\%\); n = 5) than either 1 mM AOA (\(-19.7\% \pm 3.10\%\); n = 4) or 3 mM L-Asp (\(-19.5\% \pm 3.47\%\); n = 4) alone. The addition of L-Cys up to 10 mM failed to reverse the relaxation induced by combined treatment with 1 mM AOA and 3 mM L-Asp (Figure 1F). These functional experiments suggest that CSE and 3MST, but not CSE, are major enzymes that generate H\(_2\)S in porcine LES. Supporting this conclusion, a histochemical analysis revealed the expression of CSE and 3MST, but not CSE, in the porcine LES (Figure 2). Both circular and longitudinal muscle layers showed positive staining for both enzymes. Effects of H\(_2\)S-generating enzyme inhibitors on circular smooth muscle of esophageal body were also examined. In a similar manner to LES, both AOA and L-Asp induced concentration-dependent relaxation, whereas PAG induced no relaxation. However, the extents of maximum relaxation obtained with 1 mM AOA (\(-10.9\% \pm 1.57\%\); n = 4) and 3 mM L-Asp (\(-10.4\% \pm 2.02\%\); n = 4) in esophageal body were significantly lower than those in LES, respectively (Figure 1G). In addition, contributions of L-type Ca\(^{2+}\) channels and a Rho kinase signaling pathway to the basal tone of LES were examined by using the pharmacologic inhibitors including nifedipine and Y-27632. The maximum relaxations obtained with 10 \(\mu\)M nifedipine (\(-12.4\% \pm 1.38\%\); n = 4) and 10 \(\mu\)M Y27632 (\(-7.1\% \pm 2.76\%\); n = 4) were significantly lower than that (\(27.4\% \pm 2.55\%\); n = 5) with 1 mM AOA and 3 mM L-Asp.

NaHS exhibited a biphasic response in a concentration-dependent manner (Figure 1F). At concentrations between 1 and 300 \(\mu\)M, NaHS caused relaxation, with the maximal relaxation of \(-13.3\% \pm 4.11\%\) being obtained at 300 \(\mu\)M. At higher concentrations, the relaxant effect of NaHS was attenuated, and the level of force gradually increased in a concentration-dependent manner. The level of force obtained with 10 mM NaHS (3.0\% \pm 2.63\%; n = 6) was similar to the resting level (Figure 1H). The NaHS-induced relaxation was inhibited by TEA, a nonselective K\(^+\) channel blocker. The extent of the relaxation induced by 300 \(\mu\)M NaHS in the presence of 1 mM TEA was \(-4.8\% \pm 1.41\%\) (n = 4).

**Effects of the Extracellular Na\(^{+}\) Concentration ([Na\(^{+}\)]\(i\)) and Strophanthidin on Amino-oxyacetic Acid+L-Aspartic Acid–Induced Relaxation**

The relaxant effect of AOA and L-Asp was evident under resting conditions. Therefore, whether or not AOA and L-Asp also induced relaxation under a contracted state was investigated (Figure 4). For this purpose, isotonic 118-KES (25.3 mM Na\(^{+}\)) was used to induce a contracted state. 118-KES induced an initial transient contraction (100%; n = 4), followed by a small sustained contraction (37 \pm 5.2%; n = 4) in porcine LES circular muscle strips (Figure 4A). The application of 1 \(\mu\)M nifedipine during the sustained contraction completely eliminated the contraction, restoring it to the resting level (Figure 4A). However, AOA+L-Asp failed to induce any relaxation during the sustained contraction induced by the isotonic 118-KES (Figure 4B). This observation is apparently inconsistent with that obtained under resting conditions in 137-NES. The lower concentration of Na\(^{+}\) in the isotonic 118-KES might have inhibited the relaxant effect of AOA+L-Asp. Therefore, the effect of AOA+L-Asp was examined during contraction induced by hypertonic 118-KES, which contains 137.4 mM Na\(^{+}\) (Figure 4C). Hypertonic 118-KES also induced an initial transient contraction followed by a sustained contraction (Figure 4C). The level of force at the peak of the initial transient contraction (92\% \pm 6.2\%; n = 4) was comparable with that seen with isotonic 118-KES, whereas the level of force of the sustained phase (59\% \pm 4.6\%; n = 4) was significantly greater than that obtained with isotonic 118-KES. Application of 1 mM AOA and 3 mM L-Asp during the sustained phase of contraction induced by the hypertonic 118-KES resulted in significant relaxation (Figure 4C). The extent of this relaxation (\(-16\% \pm 3.6\%; n = 4\) was slightly, but significantly, smaller than that seen under resting conditions (Figure 1F).

Next, we examined the effects of reducing the extracellular Na\(^{+}\) concentration ([Na\(^{+}\)]\(i\)) on the relaxant effect of AOA+L-Asp under resting conditions (Figure 4D and E). The ES with lower concentrations of Na\(^{+}\) was prepared by replacing Na\(^{+}\) with equimolar concentrations of Tris-HCl (pH 7.3) to maintain the isotonicity. Changing the bathing solution from 137-NES...

### Amino-oxyacetic Acid+L-Aspartic Acid–Induced Relaxation Was Accompanied by a Decrease in [Ca\(^{2+}\)]\(i\)]

Because the maximum relaxation was obtained by combined treatment with 1 mM AOA and 3 mM L-Asp, this combined treatment (AOA+L-Asp) was used as the standard protocol in subsequent investigations to elucidate the mechanism underlying the relaxation. First, we examined the effects of TTX, L-NAME (an nitric oxide synthase inhibitor), and TEA on AOA+L-Asp–induced relaxation. As shown in Figure 3, the extent of AOA+L-Asp–induced relaxation obtained in the presence of 1 \(\mu\)M TTX (\(-25.1\% \pm 4.65\%; n = 4\)), 10 \(\mu\)M L-NAME (\(-28.2\% \pm 3.11\%; n = 4\)), and 1 mM TEA (\(-30.4\% \pm 4.54\%; n = 4\)) did not significantly differ from that seen in their absence (\(-27.4\% \pm 2.55\%; n = 5\)). However, the simultaneous monitoring of [Ca\(^{2+}\)]\(i\) and force revealed that AOA+L-Asp–induced relaxation was accompanied by a significant decrease in [Ca\(^{2+}\)]\(i\) (Figure 3D). The levels of [Ca\(^{2+}\)]\(i\) and force during AOA+L-Asp–induced relaxation were \(-42.3\% \pm 2.9\%\) and \(-13.5\% \pm 4.6\%\), respectively, with the levels of [Ca\(^{2+}\)]\(i\), obtained at rest and at peak contraction induced by 10 \(\mu\)M CCh set at 0% and 100%, respectively.
to 40-ES, which contains 40 mM Na\(^{+}\), induced a sustained increase in \([\text{Ca}^{2+}]\) and contraction (Figure 5A). The subsequent application of 1 mM AOA and 3 mM L-Asp induced relaxation (Figure 4D); however, the extent of this relaxation was substantially smaller than that seen in 137-NES (Figure 1F). The relaxant effect of AOA+L-Asp was reduced in a concentration-dependent manner by lowering the extracellular [Na\(^{+}\)] (Figure 4E).

Strophanthidin is an inhibitor of Na\(^{+}/K^{+}\) ATPase. Therefore, it was used to reduce the Na\(^{+}\) gradient across the plasma membrane by increasing the intracellular [Na\(^{+}\)]. The application of 50 \(\mu\)M strophanthidin induced a transient

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**Figure 2. Microscopy images of immunohistochemistry for H\(_2\)S-generating enzymes in porcine LES.** LES tissue was stained by (A) mouse CBS monoclonal antibody, (B) rabbit 3MST polyclonal antibody, and (C) rabbit CSE polyclonal antibody. (D) Tissue stained only by anti-mouse IgG antibody. (E) Tissue stained only by anti-rabbit IgG antibody. Microscopic images were obtained with a \(\times40\) objective lens (Nikon, Tokyo Japan), and images were captured with a CCD camera (Nikon) and saved as JPEG files.
elevation of force during the resting state obtained with 137-NES (Figure 4F), accompanied by a transient increase in \([\text{Ca}^{2+}]_i\) (Figure 5B). This was followed by sustained relaxation (Figure 4F). The subsequent application of 1 mM AOA and 3 mM L-Asp failed to induce any relaxation (Figure 4F).

### Discussion

Since the identification of the enzymatic mechanism underlying \(\text{H}_2\text{S}\) production,\(^{24}\) several studies have been
performed focusing on the physiological and/or pathophysiological roles of H₂S in the gastrointestinal tract.²⁵ In the present study, we showed for the first time that endogenous H₂S contributes to myogenic tone regulation of the LES. Endogenous H₂S maintains the basal [Ca²⁺]i level via regulation of NCX, thereby contributing to the generation of the basal tone in the LES.

Endogenous generation and release of H₂S have been shown in intact and living muscle layers of the mouse colon.²⁶ Because H₂S is generated and consumed inside cells,⁹ it is appropriate to use H₂S-generating enzyme inhibitors to investigate effects of endogenous H₂S on smooth muscle contractility. Therefore, to determine the role of endogenous H₂S in the regulation of porcine LES contractility, we used pharmacologic inhibitors of H₂S-generating enzymes. We found that the H₂S-generating enzymes CBS and 3MST were responsible for endogenous H₂S production. The expression of CBS and 3MST in the porcine LES was confirmed by immunohistochemistry (Figure 2). Although millimolar concentrations of pharmacologic inhibitors for CBS and 3MST were used, their relaxant effects were, at least in part, reversed by an H₂S substrate. These observations support the specificity of the effect of the pharmacologic inhibitors. Similar millimolar concentrations of AOA were also used in another study, which suggested that AOA-sensitive endogenous H₂S helps maintain contractility in mouse gastric smooth muscle.²⁷

Many studies have used the H₂S donor NaHS to investigate the physiological and/or pathophysiological roles of H₂S.¹⁴,²⁷,²⁸ In one study, it was shown that NaHS induced biphasic duodenal contraction and relaxation in rats.¹⁴ The initial contraction was mediated by activating transient receptor potential vanilloid 1 in afferent nerve terminals, followed by the release of substance P.
Subsequent relaxation was mediated by activating K<sub>ATP</sub> channels. Another study showed that NaHS inhibited spontaneous contractions of rat colon via direct inhibition of L type Ca<sup>2+</sup> channels. Furthermore, it was shown that NaHS increased the murine gastric basal tone at low concentrations (<100 μM) but decreased it at high concentrations (>100 μM). The effects of NaHS on smooth muscle contractility seem to be variable, depending on the types of tissues and NaHS concentration. The present study observed a biphasic effect of NaHS, with a relaxant effect at lower concentrations (1–300 μM) and a contractile effect at higher concentrations. The relaxant effect was sensitive to TEA, suggesting the involvement of K<sup>+</sup> channels. H<sub>2</sub>S is usually metabolized so effectively inside the cells that a gradient of H<sub>2</sub>S concentration can develop across the cytoplasmic membrane of smooth muscle cells. The intracellular H<sub>2</sub>S concentration can reach a level much lower than that of the ES. Therefore, to mimic endogenous H<sub>2</sub>S action, considerably high concentrations of NaHS were required in the present study.

We therefore propose the following hypothesis: (1) H<sub>2</sub>S affects K<sup>+</sup> channels and NCX exclusively outside and inside the cell membrane, respectively; (2) at concentrations <300 μM, NaHS only activates the K<sup>+</sup> channel, whereas at ≥300 μM, it may activate not only the K<sup>+</sup> channels but also NCX; and (3) endogenously generated H<sub>2</sub>S only affects NCX inside the cell membrane. The H<sub>2</sub>S enzyme inhibitors, therefore, affected only the function of NCX and not that of K<sup>+</sup> channels.

AOA+L-Asp–induced relaxation was resistant to TTX. The enteric nerves are suggested to play a negligible role, if any, in the maintenance of the basal LES tone by endogenous H<sub>2</sub>S. The observations that the AOA+L-Asp–induced relaxation was resistant to either L-NAME or TEA suggest the absence of involvement of nitric oxide or K<sup>+</sup> channels. Decreased [Ca<sup>2+</sup>]<sub>i</sub> in AOA+L-Asp–induced relaxation suggested the possible involvement of L-type Ca<sup>2+</sup> channels as previous studies showed, but this was not applied to porcine LES in the present study, because an L-type Ca<sup>2+</sup> channel blocker had no effect on AOA+L-Asp–induced relaxation (Figure 4). The difference in [Na<sup>+</sup>] between the 137-NES and isotonic 118-KES suggested that extracellular [Na<sup>+</sup>] was a key factor in AOA+L-Asp–induced response, implying the involvement of NCX.

NCX plays a critical role in the regulation of the contraction of cardiac muscle, vascular smooth muscle, and visceral smooth muscles, such as stomach, uterus, and ureter. NCX transports Ca<sup>2+</sup> across the membrane bidirectionally depending on the gradient of the electrochemical potential of Na<sup>+</sup>. The changes in intracellular or extracellular [Na<sup>+</sup>] are key factors in regulation of NCX. In the present study, either decreasing extracellular [Na<sup>+</sup>] by replacing Na<sup>+</sup> with Tris-HCl or increasing intracellular [Na<sup>+</sup>] by strophanthidin led to an increase in [Ca<sup>2+</sup>]<sub>i</sub>. These observations suggest the functional involvement of NCX in the regulation of the basal level of [Ca<sup>2+</sup>]<sub>i</sub> in porcine LES smooth muscle. In contrast, the effect of H<sub>2</sub>S-generating enzyme inhibitors was significantly inhibited by either lowering [Na<sup>+</sup>]<sub>i</sub> or increasing [Na<sup>+</sup>]<sub>i</sub>. Furthermore, the decrease in the resting level of [Ca<sup>2+</sup>]<sub>i</sub>, induced by H<sub>2</sub>S-generating enzyme inhibitor was significantly augmented in NCX TG mice (Figure 6). These findings collectively suggest

![Figure 5](image-url)
Figure 6. Enhancement of AOA-induced decrease in \([\text{Ca}^{2+}]_\text{i}\) in NCX TG mice antral smooth muscle sheets. (A–C) Representative recordings (A, B) and summary (C) of the AOA-induced decrease in \([\text{Ca}^{2+}]_\text{i}\) in wild-type mice (n = 5) and NCX mice (n = 4), respectively. In C, the level of \([\text{Ca}^{2+}]_\text{i}\) obtained at the peak elevation induced by ionomycin and that obtained in the \(\text{Ca}^{2+}\)-free solution were assigned values of 100% and 0%, respectively. The net responses are each expressed as a percentage of this value. Data are means ± SEM (n = 4). **P < .01 versus control.
that endogenous H2S maintains the basal level of [Ca2+]i, in an NCX-dependent manner, thereby contributing to the generation of the basal tone of LES.

Nevertheless, how H2S regulates the activity of NCX to maintain resting [Ca2+]i level in smooth muscle remains to be investigated. It is also unclear whether H2S directly or indirectly regulates the NCX activity. NCX is known to be functionally linked to Na+/K+-ATPase function and increased [Na+]i in lung epithelial cells and renal tubular epithelial cells.

Increasing the [Na+]i inhibits the forward mode activity of NCX, resulting in an increase in the net Ca2+ inflow. Therefore, endogenous H2S may inhibit Na+/K+-ATPase, thereby indirectly potentiating the reverse mode of NCX (Figure 7).

The H2S/NCX pathway is extremely important for the generation of the smooth muscle basal tone, especially in LES. Indeed, AOA and L-Asp but not PAG induced the sustained relaxation of circular smooth muscle strips of esophageal body in a similar manner to LES. However, the extent of maximum relaxation obtained with AOA and L-Asp in the esophageal body were significantly lower than in LES (Figure 1G). The contribution of H2S-producing enzymes to the basal tone of LES was much greater than that to the basal tone of the esophageal body. However, it has been reported that both L-type Ca2+ channels and a Rho kinase signaling pathway are involved in the basal tone of LES. We compared the relative contribution of L-type Ca2+ channels, a Rho kinase signaling pathway, and endogenous H2S with the basal tone of LES with corresponding inhibitors. The contribution of endogenous H2S was much greater than those of L-type Ca2+ channels and the Rho kinase signaling pathway, indicating that endogenous H2S plays a central role in the maintenance of the basal tone in LES.

In conclusion, endogenous H2S maintains the basal level of [Ca2+]i by promoting the reverse mode of NCX in porcine LES, thereby contributing to the myogenic generation of basal tone. H2S may be a key factor in the regulation of LES contractility. Because dysfunction of LES contractility underlies the pathogenesis of gastroesophageal reflux disease and esophageal motility disorders, H2S/NCX may be a target for novel treatments for these diseases. Further basic and clinical research is required to clarify the roles of H2S and potentially provide new treatment strategies.

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Correspondence
Address correspondence to: Eikichi Ihara, MD, PhD, Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan. e-mail: eikichi@intmed3.med.kyushu-u.ac.jp; fax +81-92-642-5287.

Author contributions
Xiaopeng Bai, Yoshimasa Tanaka, Eikichi Ihara, and Katsuya Hirano designed this study. Xiaopeng Bai and Yoshimasa Tanaka performed the physiological experiments. Kayoko Nakano, Mayumi Hirano, Yoshinao Oda, Satomi Kita, Takahiro Iwamoto, and Kazuhiro Nakamura provided technical and material supports. Xiaopeng Bai, Eikichi Ihara, and Kayoko Nakano wrote the manuscript. Katsuya Hirano, Kayoko Nakano, and Yoshihiro Ogawa supervised this study.

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The authors disclose no conflicts.

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