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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

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

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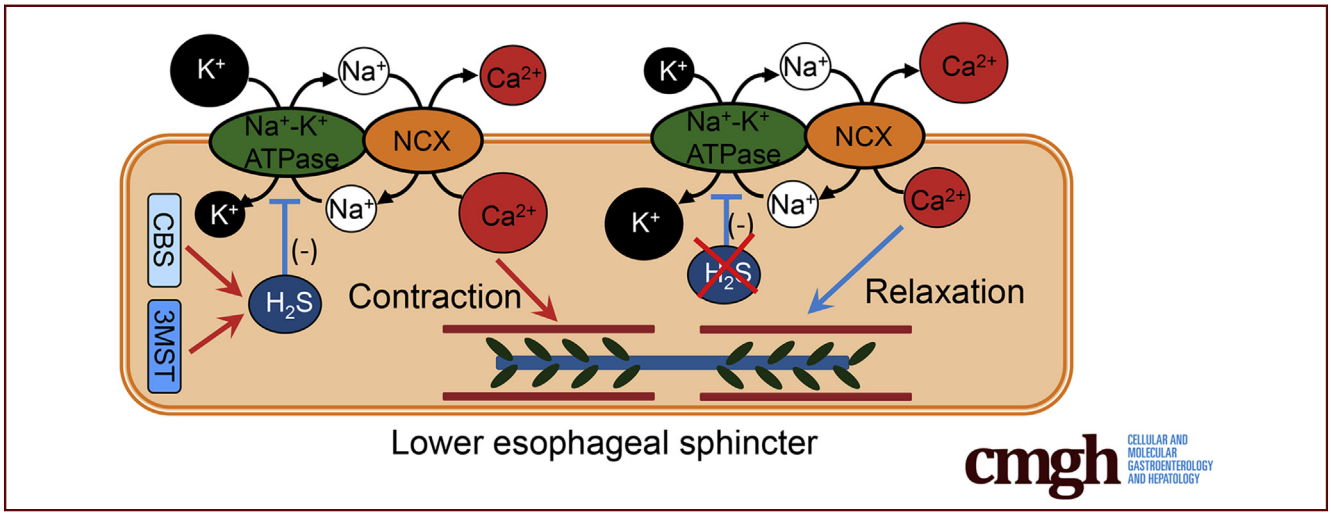

SUMMARY

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_2S) 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 ($[Ca^{2+}]_i$) and force were simultaneously monitored in fura-2-loaded strips with front-surface fluorometry. The contribution of endogenous H_2S to LES contractility was investigated by examining the effects of inhibitors of H_2S-generating enzymes, including cystathionine-β-synthase, cystathionine-γ-lyase, and 3-mercaptopyruvate sulfurtransferase, on the LES function.

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 $[Ca^{2+}]_i$ and inversely correlated with the extracellular Na^+ concentration ($[Na^+]_o$)

(25-137.4 mM), indicating involvement of an Na^+/Ca^{2+} exchanger. The reduction in the basal $[Ca^{2+}]_i$ level by AOA was significantly augmented in the antral smooth muscle sheets of Na^+/Ca^{2+} exchanger transgenic mice compared with wild-type mice.

CONCLUSIONS: Endogenous H_2S regulates the LES myogenic tone by maintaining the basal $[Ca^{2+}]_i$ via Na^+/Ca^{2+} exchanger. H_2S-generating enzymes may be a potential therapeutic target for esophageal motility disorders, such as achalasia. *(Cell Mol Gastroenterol Hepatol 2018;5:209–221; https://doi.org/10.1016/j.jcmgh.2017.11.004)*

Keywords: Lower Esophageal Sphincter; Myogenic Tone Regulation; Hydrogen Sulfate; Na^+/Ca^{2+} Exchanger.

The lower esophageal sphincter (LES) is a region of circular smooth muscle that possesses basal tone and functions as a barrier at the esophagogastric junction. The LES tone is primarily myogenic in origin and is regulated myogenically and neurogenically on stimulation.[1] Dysfunction of LES contractility underlies the pathogenesis of clinically important diseases, including gastroesophageal reflux disease and motility disorders represented by achalasia. Understanding the mechanisms controlling LES tone is crucial for gaining insight into the treatment of these diseases.

Control of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and Ca^{2+} sensitivity of the contractile apparatus play a key role in myogenic regulation of the LES tone. The activation of the L-type Ca^{2+} channel and Rho-associated kinase contribute to maintaining the myogenic tone by increasing $[Ca^{2+}]_i$ and Ca^{2+} sensitivity, respectively.[2,3] The LES tone is also neurogenically regulated by excitatory and inhibitory vagal pathways.[4,5] The excitatory vagal pathway is mediated by cholinergic neurons, whereas the inhibitory vagal pathway is mediated by the nonnoradrenergic/noncholinergic neurons. The gasotransmitters nitric oxide and carbon monoxide act as neurotransmitters in the inhibitory pathway.[6] Hydrogen sulfide (H_2S) has been identified as a third gasotransmitter[7,8]; however, its role in the regulation of the LES tone remains unclear.

H_2S 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-cystein (L-Cys) for H_2S 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.[9–13] H_2S carries out various physiologic functions in different tissues, including gastrointestinal smooth muscle. It opens ATP-sensitive K^+ channels (K_{ATP} channels) to control gastrointestinal contractility.[14] H_2S also stimulates transient receptor potential vanilloid 1 channels in rat duodenum, causing duodenal smooth muscle contraction via the local release of substance P.[15] 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 H_2S.[14,16] However, the role of H_2S in the regulation of LES contractility in either physiological or pathologic situations remains largely unknown.

The present study examined the role of endogenous H_2S in the regulation of the basal tone of LES. NaHS, a stable H_2S donor, has been used to examine the effects of exogenous H_2S,[17,18] whereas inhibitors of H_2S-generating enzymes have been used to investigate the functional role of endogenous H_2S.[16,19] In the present study, the expression of 3 major H_2S-generating enzymes in porcine LES was determined and the effects of H_2S-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 layer, the circular smooth muscle sheets of the LES were excised and cut into strips (5 × 2 mm) under a binocular microscope.

Na^+/Ca^{2+} Exchanger Transgenic Mice and Tissue Preparation of Antral Smooth Muscle Sheets

It has been shown that H_2S-generating enzyme CBS plays a role in myogenic tone generation in mouse antral smooth muscle.[16] Na^+/Ca^{2+} exchanger (NCX) 1.3 transgenic (TG) mice were used to determine the association of endogenous H_2S with NCX. NCX1.3 TG mice in a C57BL/6J background were generated as previously described.[20] TG mice were

Abbreviations used in this paper: **AOA, amino-oxyacetic acid; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CBS, cystathionine-β-synthase; CCh, carbachol; CSE, cystathionine-γ-lyase; ES, extracellular solution; H_2S, hydrogen sulfide; K_{ATP} channels, ATP-sensitive K^+ channels; KES, K^+ extracellular solution; L-Asp, L-aspartic acid; L-Cys, L-cysteine; LES, lower esophageal sphincter; L-NAME, N^{ω}-nitro-L-arginine methyl ester; $[Na^+]_o$, extracellular Na^+ concentration; NCX, Na^+/Ca^{2+} exchanger; NES, normal extracellular solution; 3MST, 3-mercaptopyruvate sulfurtransferase; 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 mm^2) 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 K^+ 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 $[Ca^{2+}]_i$ in porcine LES circular muscle strips and mouse antral smooth muscle sheets were monitored using fura-PE3 front-surface fluorimetry. 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 acetoxymethyl ester (fura-PE3/AM), 250 nM probenecid, and 5% fetal bovine serum for 90 minutes at 37°C under aeration with 5% CO_2 and 95% O_2.[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-Ca^{2+} 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 (F_{340}) and 380 nm (F_{380}) excitation and their ratio (F_{340}/F_{380}) were continuously monitored.[22] In porcine LES circular muscle strips, changes in $[Ca^{2+}]_i$ and force were simultaneously monitored. Carbachol (CCh) induced stable and reproducible responses in porcine LES circular muscle strips. Therefore, the levels of $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ induced by 50 μM ionomycin and subsequent incubation in Ca^{2+}-free solution containing 0.5 mM ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid were recorded at the end of each experimental protocol. The level of $[Ca^{2+}]_i$ obtained at peak elevation induced by ionomycin and that obtained in the Ca^{2+}-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 $\times$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 $CaCl_2$, 1.2 $MgCl_2$, 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 $CaCl_2$, 1.2 $MgCl_2$, 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). N^{ω}-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_2S in the generation of basal tone of LES, we examined the effects of inhibitors of 3 H_2S-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 1*A* and *B*). The maximum relaxation obtained with 1 mM AOA and 3 mM

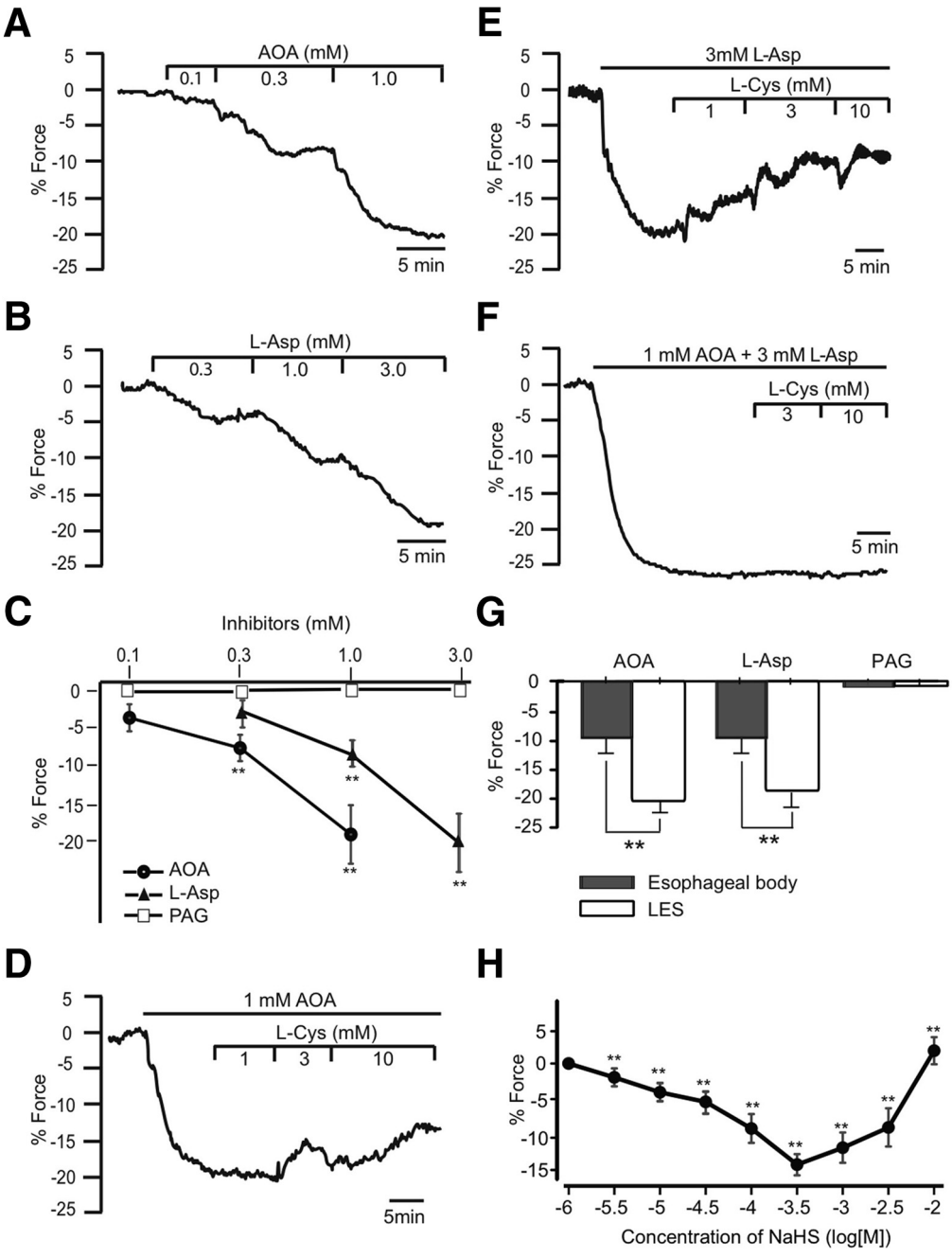

Figure 1. The relaxant effect of inhibitors of H_2S-generating enzymes in porcine LES circular smooth muscle. (*A*, *B*) Representative recordings of the relaxant responses induced by AOA (*A*, n = 4) and L-Asp (*B*, n = 4). (*C*) Concentration-dependent responses to inhibitors of H_2S-generating enzymes (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% ± 3.10% (n = 4) and −19.5% ± 3.47% (n = 4) (Figure 1*C*). However, PAG, an inhibitor of CSE, induced no relaxation even at 3 mM (Figure 1*C*). 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_2S (Figure 1*D* and *E*). The extent of contraction induced by 10 mM L-Cys reached 4.8% ± 1.38% (n = 4) and 12.0% ± 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% ± 2.55%; n = 5) than either 1 mM AOA (−19.7% ± 3.10%; n = 4) or 3 mM L-Asp (−19.5% ± 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 1*F*). These functional experiments suggest that CSB and 3MST, but not CSE, are major enzymes that generate H_2S in porcine LES. Supporting this conclusion, a histochemical analysis revealed the expression of CSB 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_2S-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% ± 1.57%; n = 4) and 3 mM L-Asp (−10.4% ± 2.02%; n = 4) in esophageal body were significantly lower than those in LES, respectively (Figure 1*G*). 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 μM nifedipine (−12.4% ± 1.38%; n = 4) and 10 μM Y27632 (−7.1% ± 2.76%; n = 4) were significantly lower than that (27.4% ± 2.55%; n = 5) with 1 mM AOA and 3 mM L-Asp.

NaHS exhibited a biphasic response in a concentration-dependent manner (Figure 1*H*). At concentrations between 1 and 300 μM, NaHS caused relaxation, with the maximal relaxation of −13.3% ± 4.11% being obtained at 300 μM. 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% ± 2.63%; n = 6) was similar to the resting level (Figure 1*H*). The NaHS-induced relaxation was inhibited by TEA, a nonselective K^+ channel blocker. The extent of the relaxation induced by 300 μM NaHS in the presence of 1 mM TEA was −4.8% ± 1.41% (n = 4).

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 μM TTX (−25.1% ± 4.65%; n = 4), 10 μM L-NAME (−28.2% ± 3.11%; n = 4), and 1 mM TEA (−30.4% ± 4.54%; n = 4) did not significantly differ from that seen in their absence (−27.4% ± 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 3*D*). The levels of $[Ca^{2+}]_i$ and force during AOA+L-Asp–induced relaxation were −42.3% ± 2.9% and −13.5% ± 4.6%, respectively, with the levels of $[Ca^{2+}]_i$ obtained at rest and at peak contraction induced by 10 μM CCh set at 0% and 100%, respectively.

Effects of the Extracellular Na^+ Concentration ($[Na^+]$) 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 ± 5.2%; n = 4) in porcine LES circular muscle strips (Figure 4*A*). The application of 1 μM nifedipine during the sustained contraction completely eliminated the contraction, restoring it to the resting level (Figure 4*A*). However, AOA+L-Asp failed to induce any relaxation during the sustained contraction induced by the isotonic 118-KES (Figure 4*B*). 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 4*C*). Hypertonic 118-KES also induced an initial transient contraction followed by a sustained contraction (Figure 4*C*). The level of force at the peak of the initial transient contraction (92% ± 6.2%; n = 4) was comparable with that seen with isotonic 118-KES, whereas the level of force of the sustained phase (59% ± 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 4*C*). The extent of this relaxation (−16% ± 3.6%; n = 4) was slightly, but significantly, smaller than that seen under resting conditions (Figure 1*F*).

Next, we examined the effects of reducing the extracellular $[Na^+]$ on the relaxant effect of AOA+L-Asp under resting conditions (Figure 4*D* 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

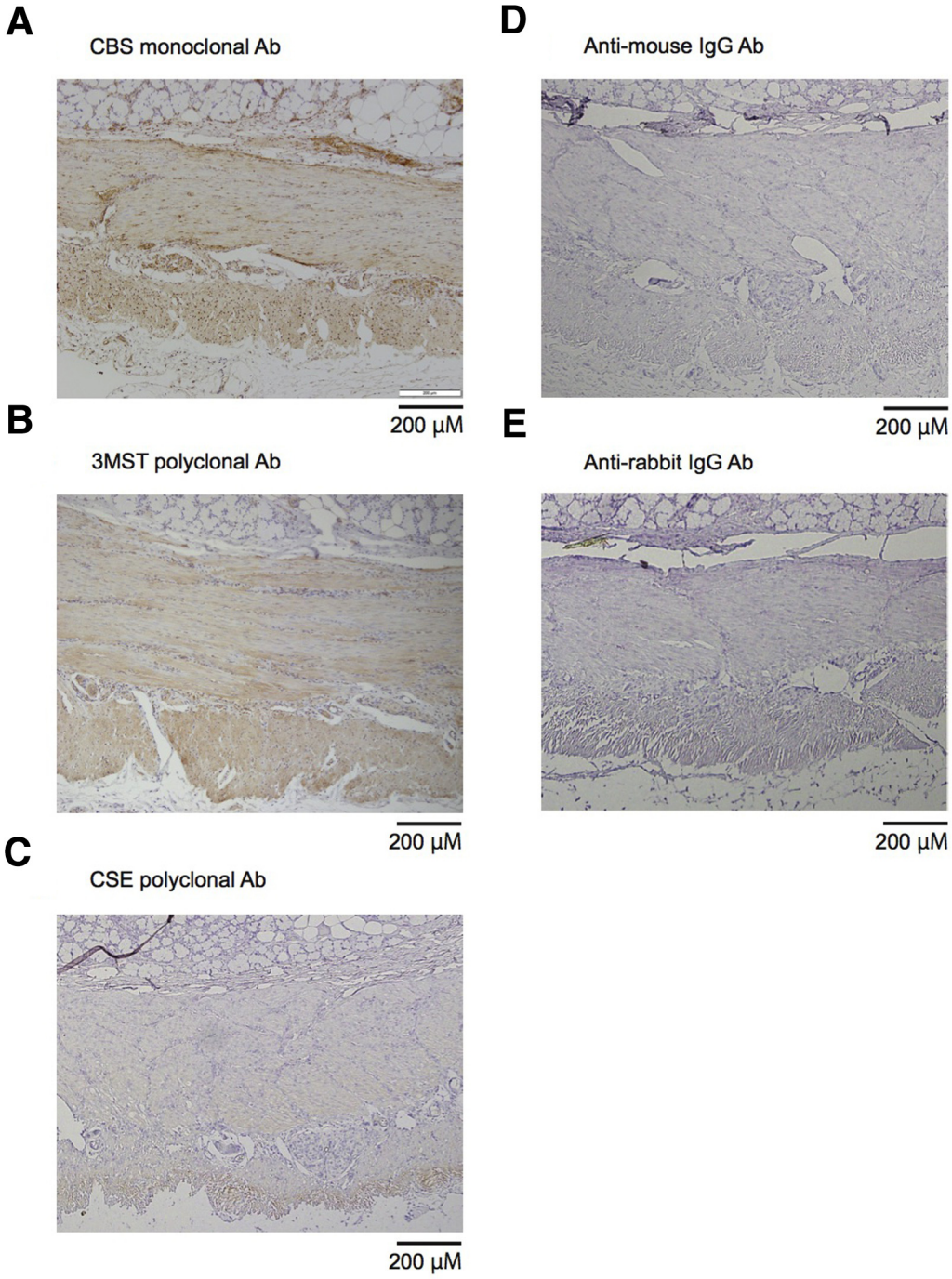

Figure 2. Microscopy images of immunohistochemistry for H_2S-generating enzymes in porcine LES. LES tissue was stained by (*A*) mouse CBS monoclonal antibody, (*B*) rabbit 3MST polyclonal antibody, and (*C*) rabbit CSE polyclonal antibody. (*D*) Tissue stained only by anti-mouse IgG antibody. (*E*) Tissue stained only by anti-rabbit IgG antibody. Microscopic images were obtained with a ×40 objective lens (Nikon, Tokyo Japan), and images were captured with a CCD camera (Nikon) and saved as JPEG files.

to 40-ES, which contains 40 mM Na^+, induced a sustained increase in $[Ca^{2+}]_i$ and contraction (Figure 5*A*). The subsequent application of 1 mM AOA and 3 mM L-Asp induced relaxation (Figure 4*D*); however, the extent of this relaxation was substantially smaller than that seen in 137-NES (Figure 1*F*). The relaxant effect of AOA+L-Asp was reduced in a concentration-dependent manner by lowering the extracellular $[Na^+]$ (Figure 4*E*).

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 μM strophanthidin induced a transient

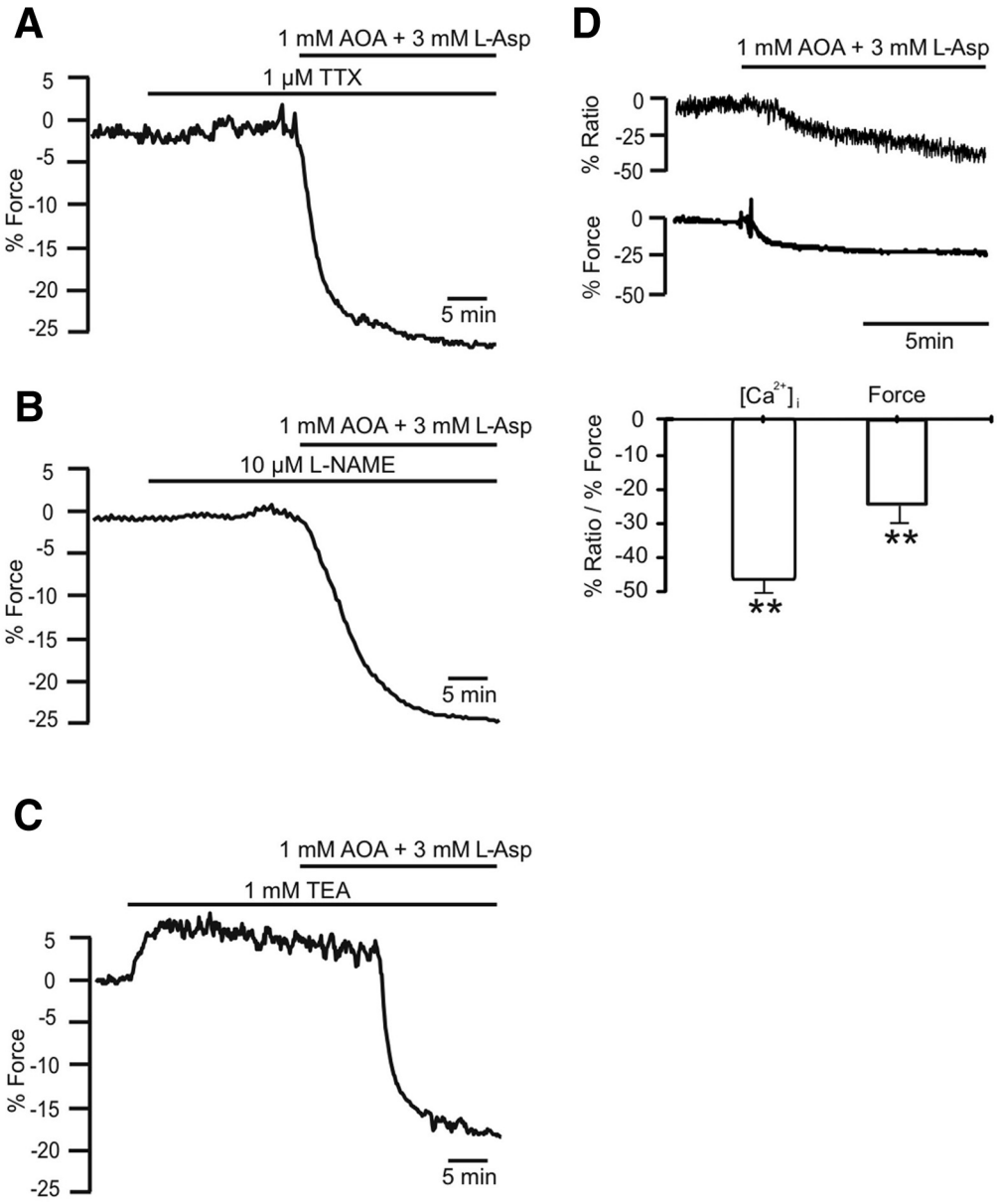

Figure 3. AOA+L-Asp–induced relaxation was accompanied by a decrease in $[Ca^{2+}]_i$. (*A–C*) Representative recordings of relaxant responses induced by AOA+L-Asp in the presence of 1 μM TTX (*A*, n = 4), 10 μM L-NAME (*B*, n = 4), and 1 mM TEA (*C*, n = 4). In *A–C*, force is expressed as %, with the levels of force obtained at rest in NES and at peak contraction induced by 118-KES set at 0% and 100%, respectively. (*D*) Representative recording and summary of levels of $[Ca^{2+}]_i$ and force changed by AOA+L-Asp application (n = 4). In *D*, the net responses are each expressed as a percentage of those observed at the peak contraction induced by 10 μM CCh as control. Data are means ± SEM (n = 4). $^{**}P < .01$ versus control.

elevation of force during the resting state obtained with 137-NES (Figure 4*F*), accompanied by a transient increase in $[Ca^{2+}]_i$ (Figure 5*B*). This was followed by sustained relaxation (Figure 4*F*). The subsequent application of 1 mM AOA and 3 mM L-Asp failed to induce any relaxation (Figure 4*F*).

Enhancement of the Amino-oxyacetic Acid–Induced Decrease in $[Ca^{2+}]_i$ in Na^+/Ca^{2+} Exchanger Transgenic Mice Antral Smooth Muscle Sheets

The findings that the AOA+L-Asp–induced relaxation was susceptible to reducing the Na^+ gradient across the plasma membrane, either by reducing the extracellular $[Na^+]$ or by treatment with strophanthidin (Figures 4 and 5), consistently suggest the involvement of NCX in the relaxation. The involvement of NCX in AOA+L-Asp–induced relaxation was further corroborated by an experiment using NCX TG mice. In the antral smooth muscle sheets of wild-type mice, AOA induced a concentration-dependent decrease in the resting level of $[Ca^{2+}]_i$ in 137-NES (Figure 6*A*). The reduction in the basal $[Ca^{2+}]_i$ level by AOA was significantly augmented in NCX TG mice (Figure 6*B* and *C*). The level of $[Ca^{2+}]_i$ obtained with 3 mM AOA in NCX TG mice (−45.4% ± 2.67%; n = 4) was significantly lower than that seen in wild-type mice (−17.5% ± 1.68%; n = 5) (Figure 6*C*).

Discussion

Since the identification of the enzymatic mechanism underlying H_2S production,[24] several studies have been

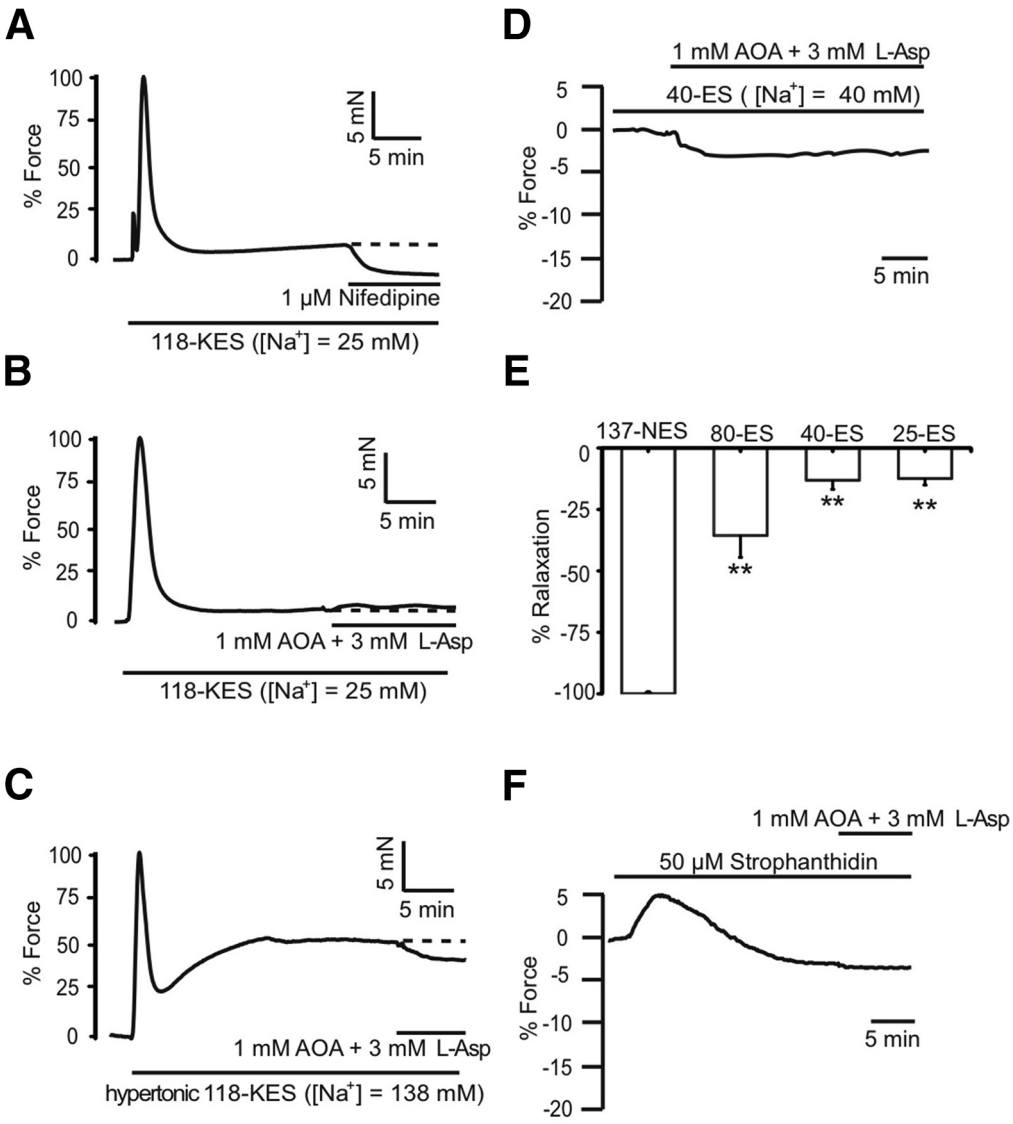

Figure 4. Effects of the extracellular Na^+ concentration ($[Na^+]$) and strophanthidin on the AOA+L-Asp–induced relaxation. (*A*, *B*) Representative recordings of relaxant responses induced by 1 μM nifedipine (*A*, n = 4) and AOA+L-Asp (*B*, n = 4) in isotonic 118-KES, respectively. (*C*, *D*) Representative recordings of relaxant responses induced by AOA+L-Asp in hypertonic 118-KES (*C*, n = 4) and 40-ES (*D*, n = 4), respectively. (*E*) Summary of relaxant responses induced by AOA+L-Asp in 137-NES (n = 4), 80-ES (n = 4), 40-ES (n = 4), and 25-ES (n = 4), respectively. (*F*) Representative recording of relaxant responses induced by AOA+L-Asp in the presence of 50 μM strophanthidin (n = 4). In *E*, the net change in force during the relaxant responses is expressed as a percentage of the 137-NES as a control response. Data are means ± SEM. $^{**}P < .01$ versus control.

performed focusing on the physiological and/or pathophysiological roles of H_2S in the gastrointestinal tract.[25] In the present study, we showed for the first time that endogenous H_2S contributes to myogenic tone regulation of the LES. Endogenous H_2S maintains the basal $[Ca^{2+}]_i$ level via regulation of NCX, thereby contributing to the generation of the basal tone in the LES.

Endogenous generation and release of H_2S have been shown in intact and living muscle layers of the mouse colon.[26] Because H_2S is generated and consumed inside cells,[9] it is appropriate to use H_2S-generating enzyme inhibitors to investigate effects of endogenous H_2S on smooth muscle contractility. Therefore, to determine the role of endogenous H_2S in the regulation of porcine LES contractility, we used pharmacologic inhibitors of H_2S-generating enzymes. We found that the H_2S-generating enzymes CBS and 3MST were responsible for endogenous H_2S production. The expression of CBS and 3MST in the porcine LES was confirmed by immunochemistry (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 H_2S 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 H_2S helps maintain contractility in mouse gastric smooth muscle.[27]

Many studies have used the H_2S donor NaHS to investigate the physiological and/or pathophysiological roles of H_2S.[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.

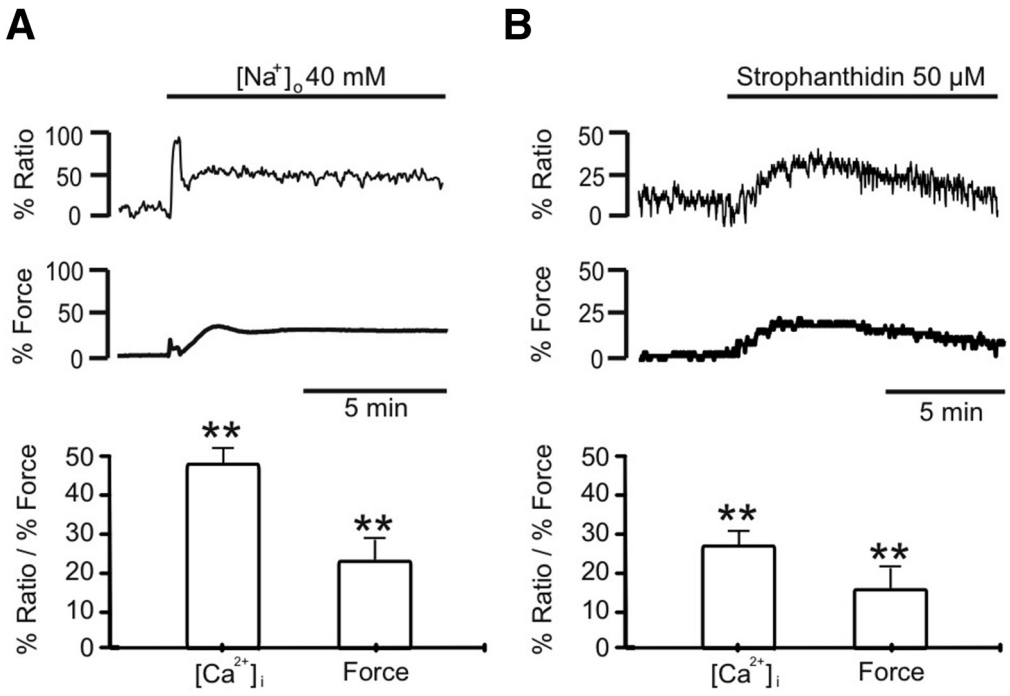

Figure 5. $[Ca^{2+}]_i$ and force levels changed by 40-ES and strophanthidin. (*A*, *B*) Representative recordings and summaries of levels of $[Ca^{2+}]_i$ and force changed by replacing 137-NES with 40-ES (n = 4) and applying strophanthidin (n = 4), respectively. The net responses are each expressed as a percentage of those observed at the peak contraction induced by 10 μM CCh as control. Data are means ± SEM. (n = 4). $^{**}P < .01$ versus control.

Subsequent relaxation was mediated by activating K_{ATP} channels.[14] Another study showed that NaHS inhibited spontaneous contractions of rat colon via direct inhibition of L type Ca^{2+} channels.[28] 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).[27] 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^+ channels. H_2S is usually metabolized so effectively inside the cells that a gradient of H_2S concentration can develop across the cytoplasmic membrane of smooth muscle cells. The intracellular H_2S concentration can reach a level much lower than that of the ES.[9] Therefore, to mimic endogenous H_2S action, considerably high concentrations of NaHS were required in the present study. We therefore propose the following hypothesis: (1) H_2S affects K^+ channels and NCX exclusively outside and inside the cell membrane, respectively; (2) at concentrations <300 μM, NaHS only activates the K^+ channel, whereas at ≥300 μM, it may activate not only the K^+ channels but also NCX; and (3) endogenously generated H_2S only affects NCX inside the cell membrane. The H_2S enzyme inhibitors, therefore, affected only the function of NCX and not that of K^+ 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_2S. 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^+ channels. Decreased $[Ca^{2+}]_i$ in AOA+L-Asp–induced relaxation suggested the possible involvement of L-type Ca^{2+} channels as previous studies showed,[28,29] but this was not applied to porcine LES in the present study, because an L-type Ca^{2+} channel blocker had no effect on AOA+L-Asp–induced relaxation (Figure 4). The difference in $[Na^+]$ between the 137-NES and isotonic 118-KES suggested that extracellular $[Na^+]$ 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,[30–32] vascular smooth muscle, and visceral smooth muscles, such as stomach, uterus, and ureter.[33–35] NCX transports Ca^{2+} across the membrane bidirectionally depending on the gradient of the electrochemical potential of Na^+.[36,37] The changes in intracellular or extracellular $[Na^+]$ are key factors in regulation of NCX. In the present study, either decreasing extracellular $[Na^+]$ by replacing Na^+ with Tris-HCl or increasing intracellular $[Na^+]$ by strophanthidin led to an increase in $[Ca^{2+}]_i$. These observations suggest the functional involvement of NCX in the regulation of the basal level of $[Ca^{2+}]_i$ in porcine LES smooth muscle. In contrast, the effect of H_2S-gernerating enzyme inhibitors was significantly inhibited by either lowering $[Na^+]_o$ or increasing $[Na^+]_i$. Furthermore, the decrease in the resting level of $[Ca^{2+}]_i$ induced by H_2S-generating enzyme inhibitor was significantly augmented in NCX TG mice (Figure 6). These findings collectively suggest

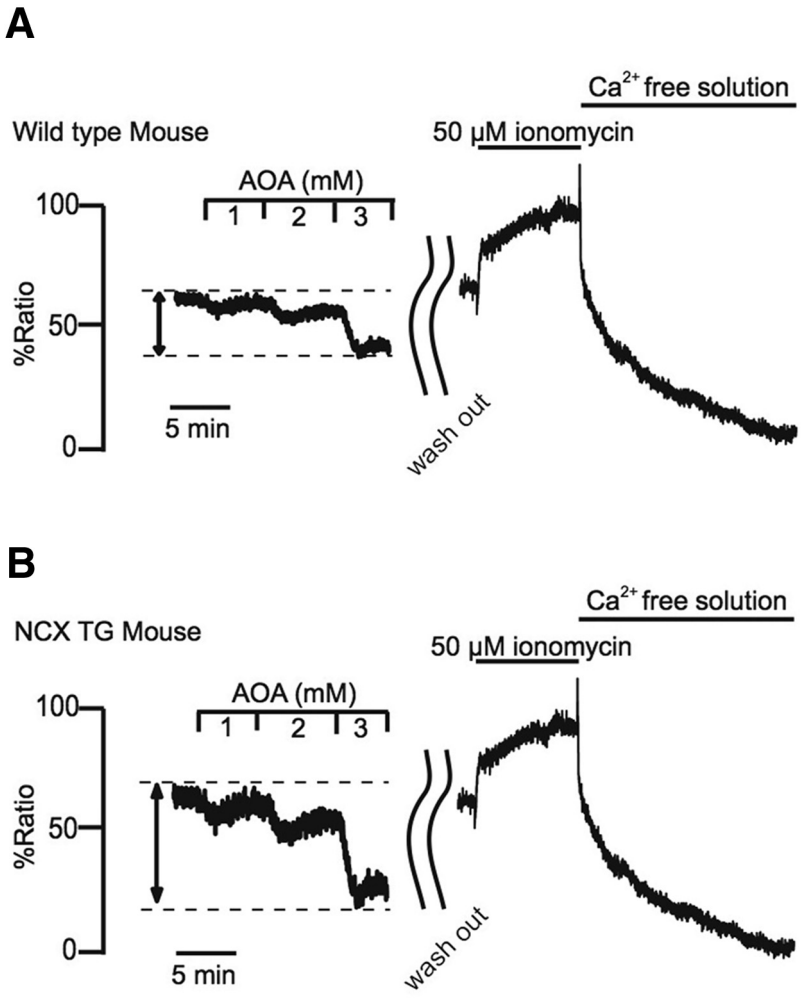

Figure 6. Enhancement of AOA-induced decrease in $[Ca^{2+}]_i$ in NCX TG mice antral smooth muscle sheets. (*A*–*C*) Representative recordings (*A*, *B*) and summary (*C*) of the AOA-induced decrease in $[Ca^{2+}]_i$ in wild-type mice (n = 5) and NCX mice (n = 4), respectively. In *C*, the level of $[Ca^{2+}]_i$ obtained at the peak elevation induced by ionomycin and that obtained in the 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.

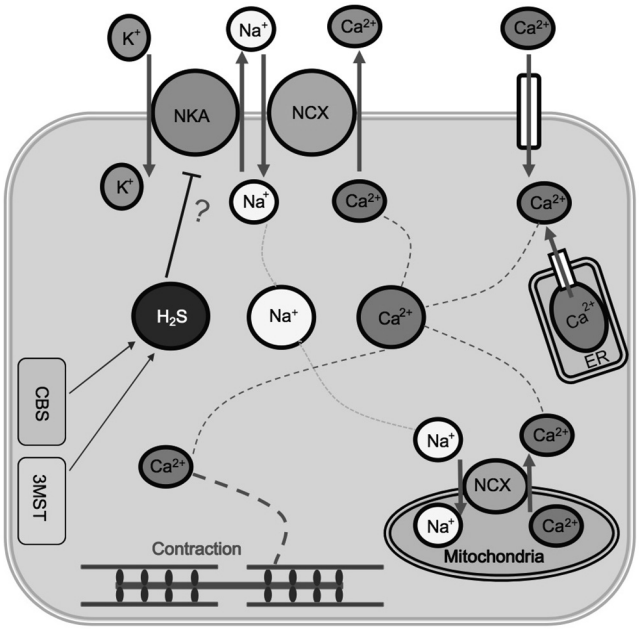

Figure 7. Proposed mechanisms of endogenous H_2S contributing to myogenic tone via regulating the NCX unit in porcine LES. H_2S is constitutively generated inside the LES circular smooth muscle cells by CBS and 3MST. Endogenous H_2S changes the balance of NCX activity via an increase in the Ca^{2+} inflow and plays a role in maintaining the $[Ca^{2+}]_i$. ER, endoplasmic reticulum; NKA, Na^+/K^+ ATPase.

that endogenous H_2S maintains the basal level of $[Ca^{2+}]_i$ in an NCX-dependent manner, thereby contributing to the generation of the basal tone of LES.

Nevertheless, how H_2S regulates the activity of NCX to maintain resting $[Ca^{2+}]_i$ level in smooth muscle remains to be investigated. It is also unclear whether H_2S directly or indirectly regulates the NCX activity. NCX is known to be functionally linked to Na^+/K^+-ATPase in smooth muscle.[34,38] It was reported that H_2S inhibited the Na^+/K^+-ATPase function and increased $[Na^+]_i$ in lung epithelial cells and renal tubular epithelial cells.[39,40] Increasing the $[Na^+]_i$ inhibits the forward mode activity of NCX, resulting in an increase in the net Ca^{2+} inflow. Therefore, endogenous H_2S may inhibit Na^+/K^+-ATPase, thereby indirectly potentiating the reverse mode of NCX (Figure 7).

The H_2S/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 1*G*). The contribution of H_2S-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 Ca^{2+} channels and a Rho kinase signaling pathway are involved in the basal tone of LES. We compared the relative contribution of L-type Ca^{2+} channels, a Rho kinase signaling pathway, and endogenous H_2S with the basal tone of LES with corresponding inhibitors. The contribution of endogenous H_2S was much greater than those of L-type Ca^{2+} channels and the Rho kinase signaling pathway, indicating that endogenous H_2S plays a central role in the maintenance of the basal tone in LES.

In conclusion, endogenous H_2S maintains the basal level of $[Ca^{2+}]_i$ by promoting the reverse mode of NCX in porcine LES, thereby contributing to the myogenic generation of basal tone. H_2S 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, H_2S/NCX may be a target for novel treatments for these diseases. Further basic and clinical research is required to clarify the roles of H_2S and potentially provide new treatment strategies.

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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 Kazuhiko 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.

Conflicts of interest
The authors disclose no conflicts.

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