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Regulation of Gastrointestinal Smooth Muscle Function by Hydrogen Sulfide

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Regestinal Smooth Muscle Function by

Hydrogen Sulfide

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science

at Virginia Commonwealth University

By

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ABSTRACT

Inhibitory neurotransmitters, chiefly nitric oxide and vasoactive intestinal peptide, cause MLC$_{20}$ dephosphorylation and muscle relaxation via inhibition of myosin light chain (MLC) kinase and activation of MLC phosphatase. Hydrogen sulfide (H$_2$S) produced as a byproduct by luminal sulfate-reducing commensal bacteria or as an endogenous signaling molecule synthesized from L-cysteine via cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) regulates muscle contraction. However, the role of H$_2$S in the regulation of MLC phosphatase activity and MLC$_{20}$ phosphorylation is not known. The aim of the present study was to examine the expression of CSE and CBS in smooth muscle cells and to elucidate the molecular mechanism of H$_2$S-induced muscle relaxation. Expression of CSE and CBS was determined by RT-PCR and western blot in muscle cells. The effect of H$_2$S on CCh-stimulated RhoA/Rho kinase pathway and muscle contraction was examined using an endogenous activator of CSE (L-cysteine) and an exogenous H$_2$S donor (NaHS). Isometric contraction in isolated muscle strips and scanning micrometry in isolated muscle cells was measured. Rho kinase activity was measured by immunokinase assay. Expression of CSE, but not CBS was detected in smooth muscle cells of stomach and colon from mouse, rabbit and human. Carbachol-induced contraction in muscle strips and in freshly dispersed muscle cells was inhibited by L-cysteine and NaHS in a concentration-dependent manner (1 to 100 mM). Glibenclamide, an inhibitor of K$_{ATP}$ channels and a known target of H$_2$S, had no effect on the inhibition of contraction by H$_2$S. L-cysteine (10 mM) or NaHS (1 mM) inhibited carbachol-induced Rho kinase activity, and the inhibition by L-cysteine was blocked in cells transfected with CSE-specific siRNA. We conclude that both endogenous and exogenous H$_2$S induce muscle relaxation, and the mechanism is inhibition of RhoA/Rho kinase activity and stimulation of MLCP activity leading to MLC$_{20}$ dephosphorylation.
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A. INTRODUCTION

H$_2$S is a colorless and flammable gas with a molecular weight of 34.08 and a vapor density (d) of 1.19, heavier than air (d=1.0). Its smell is characteristic of rotten eggs or the obnoxious odor of a blocked sewer. Its boiling point is -60.3°C, melting point is -82.3°C, and freezing point is -86°C. H$_2$S is the sulfur analog of water molecule and can be oxidized in a series of reactions to form sulfur dioxide (SO$_2$), sulfates such as sulfuric acid, and elemental sulfur.

The important life-supporting role of hydrogen sulfide (H$_2$S) has evolved from bacteria to plants, invertebrates, vertebrates, and finally to mammals. Over the years, H$_2$S had only been known for its toxicity and environmental hazard.$^1$ It has caused life destructions and extinctions on the earth over centuries. H$_2$S was believed to be the root cause for multiple mass extinctions on earth, with one of the most significant in the end of Permian period.$^2$ Since the first description of the toxicity of H$_2$S in 1713, most studies about H$_2$S have been devoted to its toxic effects. H$_2$S may be released either from inorganic sulfur compound or has its bacterial sources.$^3$ Some anaerobic organisms called green sulfur bacteria (Chlorobium) use sulfate dissolved in ocean water for respiration instead of oxygen, and subsequently produce H$_2$S.$^2$

Plants are the primary producers of organic sulfur compounds and can couple photosynthesis to the reduction of sulfate, assimilation into cysteine, and further metabolism into methionine, glutathione, and many other compounds.$^4$ They can incorporate inorganic sulfate, which is reduced to sulfide and is then incorporated into cysteine. Anionic sulfate, which is relatively abundant in the environment, is the primary sulfur source for plants. After being transported into roots, sulfate may remain in the roots or be distributed to other parts. The transportation into cells is mediated by plasma membrane-localized hydrogen/sulfate
cotransporters, driven by the electrochemical gradient established by the plasma membrane proton ATPase. Certain plants can process sulfate ion from the soil and convert it into plant protein. Subsequent decomposition of these plant proteins lead to the release of H$_2$S. This process also involves a wide variety of actinomycetes, fungi, and the bacteria heterotrophy *Proteus vulgaris*. Among those life forms that share the H$_2$S-producing ability with bacteria and plants are invertebrates. Tissue homogenates of Manila clam *Tapes philippinarum* and the lugworm *Arenicola marina* produce significant quantities of H$_2$S gas. The worm *Urechis caupo*-Fisher also produces H$_2$S.

Significant levels of H$_2$S were detected in mammalian tissues from human, rat and cow in the range of 50-160 µM. Animals have a dietary requirement for sulfur amino acids. Cysteine is the central intermediate component from which most sulfur compounds are synthesized. The enzymatic mechanisms for endogenous production of H$_2$S in mammals were also known to be mostly composed of cystathionine-β-synthase (CBS) cystathionine-γ-lyase (CSE) and 3-mercaptoppyruvate sulfurtransferase (MST). It was seen that sodium hydrogen sulfide (NaHS), a donor of H$_2$S, augmented sodium nitroprusside-induced relaxation of guinea pig ileum. It also reversed the spasmolytic effects of azide and hydroxylamine on rabbit aortic strips. In vitro studies revealed that NaHS at toxicologically relevant concentrations had complex effects on electrophysiology properties of neuronal membrane and an array of K$^+$ conductance. H$_2$S donor, NaHS, facilitated the induction of hippocampal long-term potentiation (LTP) at micromolar concentrations. This effect is beneficial, away from the conventionally assumed toxic image of H$_2$S. The expression of CBS mRNA was then confirmed in the hippocampus using Northern blot. Although it was not known by then whether altering endogenous H$_2$S level affects the LTP process, H$_2$S was labeled a "neuromodulator" in the brain. The similar approach...
It was demonstrated that both the expression and activity of CSE can be upregulated by Nitric oxide (NO), leading to increased production of H$_2$S from vascular smooth muscle cells (SMCs). Unlike NO that relaxes blood vessel walls by activating guanylyl cyclase to release cGMP, the specific molecular targets of H$_2$S in vascular SMCs are K$_{ATP}$ channels. This is the first molecule target of H$_2$S identified in the cardiovascular system. By stimulating K$_{ATP}$ channels, H$_2$S causes vasorelaxation within a physiologically relevant concentration range.

**A1. Production and metabolism of Hydrogen Sulfide in mammalian cells**

H$_2$S is generated in mammalian cells via both enzymatic and nonenzymatic pathways, although the nonenzymatic pathway only accounts for a small portion of H$_2$S production. Among enzymes involved in H$_2$S production, CBS and CSE have been extensively studied. Both the enzymes use pyridoxal 5' phosphate (vitamin B6) as a cofactor. MST along with cysteine aminotransferase (CAT) regulates endogenous H$_2$S level in specific types of cells and tissues. These enzymes are involved in transsulfuration and reverse transsulfuration pathways in different capacities and utilize specific substrates.

**Cystathionine-β-synthase (CBS).** In the presence of cysteine, more so in the presence of homocysteine, CBS catalyzes the production of H$_2$S. The most well-known reaction catalyzed by CBS is the condensation of homocysteine and serine: L serine + L-homocysteine= L- cystathionine + H$_2$O. This is the first significant step in the biosynthesis of cysteine from methionine by reverse transsulfuration. CBS catalyzes the condensation of cysteine with homocysteine to form cystathionine and H$_2$S. Binding of the allosteric activator, S-adenosyl-L-methionine (AdoMet or SAM), to the COOH domain of CBS causes a conformational change
which activates CBS. SAM is a common cosubstrate involved in methyl group transfers, transmethylation, transsulfuration, and aminopropylation pathways, and is mostly produced in liver. Deletion of the regulatory domain also can constitutively activate CBS. This regulatory domain is important for maintaining the tetrameric state of the protein. The calmodulin binding consensus sequence (19 amino acids) has also been identified in CBS at its COOH terminal domain. After calcium-activated calmodulin binds to this consensus sequence, the catalytic domain of CBS opens up and the enzyme becomes active.

CBS expression is significant in the brain is the primary enzyme that catalyzes the production of H₂S in the central nervous system. CBS was found to be highly expressed in the hippocampus and cerebellum when compared with the cerebral cortex and brain stem. In other tissues, such as cardiovascular system, respiratory system, testes, adrenal, and spleen from rats, mice or humans, CBS expression is rare or absent. In the absence of CBS, these tissues will not be able to catabolize homocysteine via transsulfuration pathway and therefore become hypersensitive to homocysteine toxicity. Another consequence for this lack of CBS is that these tissues would rely on extracellular supply of cysteine due to the deficiency in cysteine synthesis. Mutations of the regulatory domain of CBS may lead to alterations in the constitutive activation of this enzyme, leading to hereditary diseases such as in homocystinuric patients. At least 153 mutations in human CBS gene have been identified in patients with homocystinuria, characterized by an accumulation of homocysteine in the serum and urine. Both gain-of-function polymorphisms and loss-of-function polymorphism have been identified with the CBS gene.

Apart from production by CBS, H₂S production in the brain is partly regulated by testosterone and SAM. Male brains contain more H₂S than female brains at each age, suggesting the involvement of testosterone in the regulation of the H₂S level. Glucocorticoids
CBS expression is also upregulated by epidermal growth factor (EGF), transforming growth factor-β (TGF-β), cAMP, and dexamethasone in reactive astrocytes.\textsuperscript{32}

\textit{Cystathionine-γ-lyase (CSE)}. Cystathionine-γ-lyase has been conventionally abbreviated as CSE, CGL, or CTH. CSE has been described as a beta-replacing lyase with a strict specificity for the primary substrate L-cysteine and for several sulfur-containing co-substrates.\textsuperscript{33, 34} Unlike CBS which is expressed in the central nervous system, CSE is expressed abundantly in mammalian cardiovascular system and respiratory system.\textsuperscript{35} It appears to be the main \( \text{H}_2\text{S} \) producing enzyme in the liver, kidney, uterus, placenta, as well as pancreatic islets.\textsuperscript{36} Low levels of CSE are also detectable in the small intestine and stomach of rodents.\textsuperscript{37} Small amounts of CSE mRNA have also been detected in the brain.\textsuperscript{38} CSE is capable of using L-cysteine as the substrate to form two gases, \( \text{H}_2\text{S} \) and \( \text{NH}_3 \), and pyruvate.\textsuperscript{39}

The levels of \( \text{H}_2\text{S} \) in vascular tissues are affected by NO through two different mechanisms. NO increases CSE activity in vascular tissues.\textsuperscript{40} Rat aortic tissue homogenate, when incubated with a NO donor for 90 min, significantly increased \( \text{H}_2\text{S} \) generation in a concentration-dependent manner. One possibility is that NO increases the activity of cGMP-dependent protein kinase (PKG), which in turn stimulates CSE. Alternatively, NO may directly act on CSE protein. The second mechanism for NO-induced \( \text{H}_2\text{S} \) production is the upregulation of CSE expression. CSE can be upregulated by bacterial endotoxin\textsuperscript{41} as well as during liver regeneration.\textsuperscript{42} CSE is reported to be repressed during a viral infection in humans due to immunodeficiency. Some studies show that CSE is significantly unregulated in rats during the lactation period.\textsuperscript{43} CSE transcription and protein turnover rates are also affected by vitamin B6 availability.\textsuperscript{44} Stimulation of endothelial cells with vascular endothelial growth factor (VEGF)
upregulates CSE and increases H₂S release. Testosterone is another endogenous CSE stimulator. It does not change the expression of CSE but stimulates CSE activity.

Human deficiency of CSE may lead to a metabolic disorder named cystathioninuria that is inherited in an autosomal recessive manner. In patients with Cystathionuria, an excess of cystathionine is reported in the urine. Other diseases related to CSE mutation include hypercystathioninemia and increase the risk of developing atherosclerosis and bladder cancer.

**MST and CAT.** MST (or SseA) is synonymous with β-mercaptopryruvate sulfurtransferase. MST has A and B chains. MST is involved in cyanide detoxification as MST transfers the sulfane sulfur from substrate to cyanide ion, giving nontoxic thiocyanate and pyruvate. The first crystal structure of MST was derived from *Escherichia coli*. The cofactor of MST is zinc. Another enzyme that has been reported to generate H₂S in rat liver is CAT. CAT, otherwise known as cysteine aminotransferase also uses PLP (pyridoxal 5-phosphate) as the cofactor. MST and CAT are localized in both cytosol and mitochondria. MST generates H₂S in the presence of CAT and cofactors 2-oxoglutarate and PLP. CAT converts cysteine to 3-mercaptopryruvate (3-MP). MST then transfers the sulfur from 3-MP to sulfite or other sulfur acceptors or form elemental sulfur. The direct outcome of the CAT-MST pathway is the production of sulfane sulfur (or bound sulfur). H₂S would be consequently formed either through reduction of the atomic sulfur or released from thiosulfate or persulfides.

Transsulfuration and reverse transsulfuration are two opposite processes involving the interconversion between the sulfur-containing amino acids cysteine and methionine. Reverse transsulfuration occurs in vertebrates and fungi with the final product being cysteine. Transsulfuration is the transformation of cysteine into homocysteine via the intermediate L-
A significant amount of exhaled H$_2$S has been measured after intravenous administration of sodium sulfide.$^{52}$ An increased amount of exhaled H$_2$S has also been reported after inhibition of endogenous NO synthesis. The endogenous polysulfide DADS (diallyl disulfide) increases the amount of H$_2$S exhaled, as DADS act as H$_2$S donors when they react with biological thiols such as GSH. H$_2$S is also excreted in the urine primarily as sulfate (either free sulfate or thiosulfate) and in feces and flatus unchanged as free sulfide.

In the mitochondria, H$_2$S is first oxidized to thiosulfate and then to sulfite and sulfate.$^{53}$ The formation of thiosulfate is not enzymatic, but thiosulfate conversion to sulfite and/or sulfate is catalyzed by sulfide-detoxifying enzymes. Rhodanese is a sulfide-detoxifying enzyme.$^{54}$ Methylation is another catabolic pathway for H$_2$S. While the oxidation of H$_2$S occurs mainly in mitochondria, methylation mainly takes place in the cytosol.

The methylation of H$_2$S yields methanethiol (CH$_3$SH). H$_2$S has been reported to be methylated to methanethiol in vitro by the intestinal mucosa of Sprague-Dawley rats. The enzyme Thiol-S-Methyltransferase (TSMT) methylates H$_2$S into methanethiol and dimethylsulfide. TSMT is a ubiquitous enzyme with the highest activity in the colonic and cecal mucosa,$^{55}$ but its activity has also been reported in the liver, lung, and kidney. Compared with sulfide oxidation, sulfide methylation is slow. H$_2$S can also be scavenged by metallo- or disulfide-containing molecules such as horseradish peroxidise, catalase, and oxidized glutathione.$^{56}$
H₂S has several features in common with the other two well known gasotransmitters (nitric oxide and carbon monoxide) in the biological systems. Like H₂S, both nitric oxide (NO) and Carbon monoxide (CO) are colorless gases and easily penetrate the biological membrane. NO is a free radical and produces the extremely toxic hydroxyl radical when it combines with superoxide. CO is a reducing agent like H₂S. With these differences and similarities, H₂S, NO, and CO elicit several effects; some of them are opposite, whereas others are quite similar.

**H₂S as a vasorelaxant:** NO, the first identified gasotransmitter, relaxes smooth muscle of various vasculatures. Substances other than NO, including low-molecular-weight S-nitrosothiol intermediates, also contribute to the relaxation of smooth muscle. CO is known to dilate different vascular tissues, from conduit arteries to resistant arteries. H₂S-induced vasorelaxation has been demonstrated in numerous types of blood vessels (such as aorta, portal vein, mesenteric artery, cerebral arteries, and vas deferens) from different species (such as rats, mice, cows, guinea pigs, sheep, and humans). H₂S relaxes small mesenteric arteries much more potently than aortic tissues. Although rat aortic and mesenteric artery tissues produce similar levels of H₂S, H₂S is nearly sixfold more potent in relaxing rat mesenteric artery beds than relaxing rat aortic tissues. The higher sensitivity of mesenteric arteries to H₂S speaks for the importance of the gas in regulating peripheral resistance. H₂S also functions as a vasodilator in cerebral circulation. Topical application of H₂S to the newborn pigs induces dilation of pial arterioles. This vasorelaxant effect of H₂S appears to be mediated by K<sub>ATP</sub> channels as glibenclamide blocks the H₂S effect. K<sub>ATP</sub> channel is the major molecular target of H₂S for its vasorelaxant effect and smooth muscle hyperpolarization.
The vasorelaxant property of this gasotransmitter gives it a role in regulating peripheral resistance and blood pressure. Reduced endogenous H₂S level, especially in blood vessel wall, would be the cause for development of hypertension. Direct evidence for the role of endogenous H₂S in blood pressure control was demonstrated in CSE knockout mice.⁶⁵ The knockout mice manifested age-dependent development of hypertension which was comparable to that of eNOS KO mice.⁶⁶ On the other hand, endothelium-dependent vasorelaxation, triggered by the activation of muscarinic cholinergic receptors on the endothelium, was severely abolished in CSE KO mice. Therefore, it can be concluded that hypertension in CSE KO mice is caused by the elimination of CSE expression in vascular tissues, especially in the endothelium.

**H₂S as a neuromodulator.** In mammalian central nervous system (CNS), CBS was found highly expressed in the hippocampus and the cerebellum.⁶⁷ CBS is mainly localized to astrocytes⁶⁸ ᵆ⁹ and microglial cells, confirmed by immunohistological studies.⁷⁰ The activation of Ca²⁺/calmodulin pathway after neuronal excitation activates CBS-based production of H₂S. H₂S has multifaceted and important effects in CNS, and can modulate neurotransmission and neuromodulation. Glutamate is an important excitatory amino acid that functions as a neurotransmitter. Glutamate in mammalian brain is known for its role in learning and memory, such as induction of long-term potentiation (LTP) and perception of pain. LTP is a memory consolidation process, which is ignited by a brief period of high-frequency presynaptic stimulation (5–100 Hz), and this initial stimulation would enhance the postsynaptic response to subsequent presynaptic stimulation for many hours/days after the high-frequency tetanus. Abnormal glutamate metabolism can lead to excitatory neuronal injury. The neurological effects of glutamate are mediated by N-methyl-D-aspartate (NMDA) receptors⁷¹ in both central and
peripheral nervous systems, with exceptions in the bone and pancreatic islet. It has been found that at physiologically relevant concentrations H₂S selectively enhances NMDA receptor-mediated currents and expedites the induction of hippocampal LTP in rats. On the contrary, high concentration of H₂S would damage brain and lead to decreased learning and memory function. In addition to activating NMDA receptors, H₂S also directly increases glutamate secretion. With high levels of NaHS stimulation, extracellular concentrations of glutamate were increased from physiological concentrations of 2–5 to 10–15 μM resulting in cell death. H₂S-induced cell neuron death was abolished by NMDA blocker MK-801 and glutamate antagonist DL-2- amino-5-phosphonovaleric acid.

Suppression of oxidative stress is another effect of H₂S in the brain. H₂S inhibits protein oxidation, HOCl-induced cytotoxicity, intracellular protein oxidation, and lipid peroxidation. It also protects brain endothelial cells from methionine-induced oxidative stress.

**H₂S and Inflammation.** H₂S is known to exert both pro-inflammatory and anti-inflammatory effects. The upregulation of CSE and the consequent increase in H₂S production induced by LPS or pro-inflammatory cytokines can be viewed as a pro-inflammatory action or as an anti-inflammatory reaction as a compensatory protection mechanism. H₂S possess an anti-proliferative effect on T-Lymphocytes and induces apoptotic death of polymorphonuclear cells. These effects limit the development of inflammation. Injection of rats with H₂S donors, NaHS and Na₂S, inhibited leukocyte infiltration and adherence to vascular endothelium, and edema formation in a hindpaw edema model. Vice versa, inhibitors of H₂S synthesis increased leukocyte adhesion, leukocyte infiltration, and edema formation. Neuroinflammation is another prominent case in point for elucidating the role of H₂S in
Inflammatory process. The activation of glial cells and release of inflammatory factors within brain, together with the recruitment of peripheral immune cells, jointly result in neuroinflammatory damage. This damage will deteriorate or lead to neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD). NaHS attenuates LPS-induced production and release of NO and TNF-α. The anti-inflammatory effect of H₂S is also remarkable in its ability to upregulate anti-inflammatory and cytoprotective genes, such as heme oxygenase (HO). H₂S-induced upregulation of HO expression in pulmonary smooth muscle cells and macrophages has been demonstrated, which is believed to be the consequence of ERK activation.

Significant experimental evidence shows that H₂S acts as a pro-inflammatory factor in various animal models, including hindpaw edema, acute pancreatitis, LPS-induced endotoxemia, and cecal ligation and puncture induced sepsis. Studies have shown that excessive activation of K<sub>ATP</sub> channels occurs in LPS-induced hypotension and hyporeactivity to vasoconstrictor agents. As H₂S is an identified endogenous opener of K<sub>ATP</sub> channels, it was speculated that abnormal synthesis or activity of H₂S may play a part in septic or endotoxic shock. It was seen that tissue H₂S formation and plasma H₂S concentrations were increased in a septic shock rat model induced by cecal ligation and puncture. NaHS treatment of these animals significantly aggravated septic inflammatory damages. H₂S has also been reported to stimulate the generation of pro-inflammatory cytokines from human monocytes.

One such mechanism is that H₂S stimulates sensory nerve endings, releasing endogenous tachykinins such as substance P (SP), calcitonin gene-related peptide (CGRP), and neurokinin A, which causes neurogenic inflammation. A number of factors are involved in determining
whether H\textsubscript{2}S is anti-inflammatory or pro-inflammatory. The concentrations and administration routes for H\textsubscript{2}S may yield different inflammatory outcomes.

**A4. Molecular mechanisms of action of Hydrogen Sulphide**

**K\textsubscript{ATP} channels.** The activation of K\textsubscript{ATP} channels by H\textsubscript{2}S is among the first identified molecular mechanisms for H\textsubscript{2}S. Recently discovered signaling mechanisms for H\textsubscript{2}S involves covalent modification of cysteine residues in proteins through S-sulfhydration, converting cysteine -SH groups to hydropersulfide (-SSH groups).\textsuperscript{98} S-sulfhydration can be explained in a comparative fashion with S-nitrosylation. S-nitrosylation occurs between NO molecules and cysteine residues of the concerned proteins.\textsuperscript{99} Striking similarities between S-sulfhydration and S-nitrosylation are seen as both involve the covalent modification of cysteines and both are reversible by reducing agents, such as dithiothreitol (DTT).\textsuperscript{100} The functional outcome of the protein modification is different. S-sulfhydration usually contributes to the increased activity of the modified proteins, but S-nitrosylation appears to decrease the activity it in most cases. The latter could be due to the shielding of critical reactive thiol groups of the proteins. Extensive experiments on vascular tissues strongly suggest that H\textsubscript{2}S-induced vasorelaxation is mainly caused by K\textsubscript{ATP} channel openings causing hyperpolarization. This notion is largely based on the ability of glibenclamide, a K\textsubscript{ATP} channel antagonist, to block the vasorelaxant effects of H\textsubscript{2}S.\textsuperscript{101} Electrophysiological study provides direct evidence that exogenous H\textsubscript{2}S increases macroscopic or unitary K\textsubscript{ATP} currents, which is blocked by glibenclamide in isolated rat aortic and mesenteric SMCs.\textsuperscript{102} Thus, H\textsubscript{2}S-induced hyperpolarization of SMC membrane is abolished by glibenclamide.
H₂S is a strong reducing agent and may easily interact with other oxidant species. Its group allows the reduction of disulfide bonds and scavenging of reactive oxygen species and nitrogen species. Direct scavenging of peroxynitrite and the reduction of its toxicity by H₂S has been reported. This effect would endow H₂S an antioxidant role to offer cytoprotection.

A5. Hydrogen Sulphide in the gastrointestinal system

Endogenous H₂S level in GI system is made up of two components. The first source is sulfate-reducing bacteria present in the lumen of the large intestine. The second source is from mammalian cells in the GI tract. H₂S is produced at 0.2–3.4 mM in the GI tract of mice and humans by the intestinal microbiota, such as the enterobacterial flora, which use endogenous sulfur-containing compounds including amino acids. Both CSE and CBS are found to be expressed in the GI tract of rats, mice and in healthy human colon. In rat ileum, CBS and CSE mRNA were detected. The gastric mucosa expresses both CSE and CBS, although CSE appears to play a major role in H₂S generation as PPG inhibits gastric H₂S formation. The rate of H₂S production in rat ileum is comparable with that of rat aortas. Lysine acetyl salicylate injection into mice (intraperitoneally) increases the endogenous production of H₂S in intestine. H₂S also participates in the regulation of various GI functions, from motility control to secretion and inflammation. Similar to its effect on vascular contractility, H₂S inhibits the motility of jejunum and colon in humans, mice, and rats. The precontracted ileum muscles from rats and guinea pigs are relaxed by NaHS in vitro. The spontaneous contraction of the isolated ileum tissues from rabbits was also inhibited by NaHS. The relaxant effect of NaHS on the spontaneous contraction of gastric smooth muscle appears to be mediated by the activation of
channels and closing of Kv channels by NaHS had also been directly demonstrated on freshly dispersed gastric antrum myocytes using the whole cell patch-clamp recording. These observations suggest that two different types of ion channels are responsible for the dual actions of H2S on gastric motility in guinea pigs. Another ion channel involved in the effect of NaHS on mouse colon is SKCa channels, since apamin at 3 μM inhibited NaHS-induced inhibition of colon motility. It is also known that H2S may also indirectly affect GI smooth muscle contractility by acting on neurons of the enteric nervous system. The presence of H2S in the mucosa/submucosa of the colon stimulates primary afferent nerve fibers, thus increasing chloride secretion in guinea pigs and humans.

A6. Gastric motility

Contractions of gastric smooth muscle serves two basic functions: ingested food is crushed, ground and mixed, liquefying it to form what is called chyme. Chyme is forced through the pyloric canal into the small intestine, a process called gastric emptying. The stomach can be divided into two regions on the basis of motility pattern: an accordion-like reservoir that applies constant pressure on the lumen and a highly contractile grinder.

The upper stomach, composed of the fundus and upper body, shows low frequency, sustained contractions that are responsible for generating a basal pressure within the stomach. Importantly, these tonic contractions also generate a pressure gradient from the stomach to small intestine and are thus responsible for gastric emptying. Interestingly, swallowing of food and consequent gastric distention inhibits contraction of this region of the stomach, allowing it to balloon out and form a large reservoir without a significant increase in pressure.
The lower stomach, composed of the lower body and antrum, develops strong peristaltic waves of contraction that increase in amplitude as they propagate toward the pylorus. These powerful contractions constitute a very effective gastric grinder; they occur about 3 times per minute in humans and 5 to 6 times per minute in dogs. Gastric distention strongly stimulates this type of contraction, accelerating liquefaction and hence, gastric emptying. The pylorus is functionally part of this region of the stomach - when the peristaltic contraction reaches the pylorus, its lumen is effectively obliterated - chyme is thus delivered to the small intestine in spurts.

Gastric motility is controlled by a very complex set of neural and hormonal signals. Smooth muscle cells lack the striated banding pattern found in cardiac and skeletal muscle, and they receive neural innervation from the autonomic nervous system. In addition, the contractile state of smooth muscle is controlled by hormones, autocrine/paracrine agents, and other local chemical signals. Smooth muscle cells also develop tonic and phasic contractions in response to changes in load or length. Regardless of the stimulus, smooth muscle cells use cross-bridge cycling between actin and myosin to develop force, and calcium ions (Ca\textsuperscript{2+}) serve to initiate contraction.\textsuperscript{116} Contraction of smooth muscle is initiated by a Ca\textsuperscript{2+}-mediated change in the thick filaments, whereas in striated muscle Ca\textsuperscript{2+} mediates contraction by changes in the thin filaments. In response to specific stimuli in smooth muscle, the intracellular concentration of Ca\textsuperscript{2+} increases and combines with the acidic protein calmodulin. This complex activates MLC kinase to phosphorylate the light chain of myosin. Cytosolic Ca\textsuperscript{2+} is increased through Ca\textsuperscript{2+} release from intracellular stores (sarcoplasmic reticulum) as well as entry from the extracellular space through Ca\textsuperscript{2+} channels (receptor-operated Ca\textsuperscript{2+} channels).
In gastrointestinal smooth muscle cells phosphorylation of Ser\textsuperscript{19} on the 20-kDa regulatory light chain of myosin II (MLC\textsubscript{20}) by Ca\textsuperscript{2+}/calmodulin-dependent myosin light-chain (MLC) kinase is essential for initiation of muscle contraction.\textsuperscript{117, 118} Excitatory neurotransmitters such as acetylcholine initiate contraction by increasing cytosolic Ca\textsuperscript{2+}, or [Ca\textsuperscript{2+}]\textsubscript{i}, and activation of Ca\textsuperscript{2+}/calmodulin-dependent MLCK.\textsuperscript{119} The initial increase in [Ca\textsuperscript{2+}]\textsubscript{i}, and MLCK activity are transient. MLC\textsubscript{20} phosphorylation and contraction, however, are sustained via inhibition of MLC phosphatase.\textsuperscript{120} Inhibition of MLCP is initiated by two RhoA-dependent pathways: one involves phosphorylation of MYPT1, the regulatory subunit of MLC phosphatase, at Thr\textsuperscript{696} by Rho kinase, and the other involves phosphorylation of the endogenous MLC phosphatase inhibitor, CPI-17, at Thr\textsuperscript{38} by PKC.\textsuperscript{121, 122}

Inhibitory neurotransmitters such as vasoactive intestinal peptide (VIP) and nitric oxide (NO) induce relaxation through the generation of cAMP and cGMP and activation of cAMP-dependent protein kinase (PKA) or cGMP-dependent protein kinase (PKG).\textsuperscript{123, 124} Inactivation of specific targets in the signaling pathways mediating contraction upon phosphorylation by cAMP-dependent protein kinase (PKA) or cGMP-dependent protein kinase (PKG) results in muscle relaxation.\textsuperscript{125} PKA and PKG act on various loci to inhibit i) MLC kinase by inhibiting IP\textsubscript{3} generation and IP\textsubscript{3}-dependent Ca\textsuperscript{2+} release, and/or stimulating Ca\textsuperscript{2+} uptake into the sarcoplasmic stores, and ii) activate MLC phosphatase by phosphorylating RhoA at Ser\textsuperscript{188} and inhibiting its ability to activate Rho kinase, phosphorylating MYPT1 at Ser\textsuperscript{695} and blocking phosphorylation at the adjacent Thr\textsuperscript{696} by Rho kinase, and/or phosphorylating telokin (at Ser\textsuperscript{13}), an endogenous MLC phosphatase stimulator.\textsuperscript{126}
Recent studies have demonstrated that hydrogen sulphide (H\textsubscript{2}S), a gaseous transmitter like NO and carbon monoxide (CO), is involved in the regulation of several physiological functions including gastrointestinal motility.\textsuperscript{127} H\textsubscript{2}S, in addition to generation by luminal sulfate-reducing commensal bacteria in the colon, is synthesized endogenously from L-cysteine via pyridoxal-5\textdaggerdbl;phosphate-dependent enzymes, cystathionine-\textgamma;lyase (CSE) and cystathionine-\textbeta;synthase (CBS). 3-mercaptopyruvate sulfurtransferase in combination with cysteine aminotransferase can also synthesize H\textsubscript{2}S from L-cysteine.\textsuperscript{128} In the gastrointestinal tract, H\textsubscript{2}S-synthesizing enzymes have been shown to be expressed by enteric neurons, interstitial cells of Cajal and epithelial cells.\textsuperscript{129} Expression of CBS and CSE is tissue specific. Reduction in the levels of H\textsubscript{2}S accompanied by hypertension and reduced endothelium-dependent relaxation of vascular muscle in mouse lacking CSE and downregulation of CSE/L-cysteine pathway in spontaneously hypertensive rats underscores the importance of endogenous H\textsubscript{2}S in the regulation of cardiovascular functions. H\textsubscript{2}S exerts its function by acting on various targets, but unlike NO and CO, it does not affect soluble guanylyl cyclase (sGC activity) and cGMP formation.\textsuperscript{130} Some of the targets include activation of K\textsubscript{ATP} channels, mitogen-activated protein kinase pathway, capsaicin-sensitive sensory neurons, T-type Ca\textsuperscript{2+} channels or interaction with NO synthase system.\textsuperscript{131}

One of the most studied mechanisms for muscle relaxation by H\textsubscript{2}S is activation of K\textsubscript{ATP} channels. H\textsubscript{2}S increased K\textsubscript{ATP} channels currents in smooth muscle cells of rat aorta and mesenteric artery. Although, it is well-established that H\textsubscript{2}S inhibits gastrointestinal motility in vivo and both agonist- and electrically-induced contractions in vitro, it is not known for certain the site of H\textsubscript{2}S biosynthesis and the mechanism of action. In guinea pig ileum and mouse stomach and colon, the inhibitory effect of H\textsubscript{2}S was not affected by the K\textsubscript{ATP} channel inhibitor
and segments of human, mouse and rat colon the inhibitory effect of H$_2$S was dependent on both glibenclamide-sensitive and apamin-sensitive K$^+$ channels. The aim of the present study is to determine the effects of endogenous released and exogenously applied H$_2$S on smooth muscle function and to identify the targets involved in mediating the effects of H$_2$S using muscle strips and isolated muscle cells from stomach and colon of rabbit and mouse. The results demonstrate that both endogenous and exogenous H$_2$S induce muscle relaxation, and the mechanism involves inhibition of Rho kinase activity and stimulation of MLCP activity leading to MLC$_{20}$ dephosphorylation.

Rationale

The role of H$_2$S in the regulation of gastrointestinal motility in vivo and in innervated muscle in vitro has been demonstrated. A direct effect of H$_2$S on smooth muscle and the mechanism of action were never reported. Hence, the aim of the present study was to examine the expression of H$_2$S synthesizing enzymes in smooth muscle and characterize the mechanism of action of H$_2$S involved in the regulation of smooth muscle function. We have tested the hypothesis that H$_2$S synthesizing enzyme(s) are present on smooth muscle and their activation results in the inhibition of muscle contraction. A greater understanding of the H$_2$S role in smooth muscle could lead to advances with important therapeutic potential in the treatment of gastrointestinal motility disorders.

B. MATERIALS AND METHODS

B1. Reagents
Antibodies for cystathionine β synthase (CBS) and cystathionine γ lyase (CSE) were obtained from proteintech (Chicago, IL). [³²P]ATP was obtained from NEN Life Sciences Products (Boston, MA). [¹²⁵I]cAMP was obtained from PerkinElmer Life Sciences (Boston, MA); Y27632 (Rho kinase inhibitor); RNAqueousTM kit was obtained from Ambion, Austin, TX; LipofectamineTM 2000 transfection reagent, SuperScriptTM II Reverse Transcriptase kit were obtained from Invitrogen, Carlsbad, CA; PCR reagents were obtained from Applied Biosystems, Roche. CSE SiRNA and Negative siRNA were obtained from Ambion Life Technologies, Austin, TX. Collagenase CLS type II and soybean trypsin inhibitor for cell isolation were obtained from Worthington, Freehold, NJ; Western blotting materials and protein assay kit, Tris-HCl ready gels were obtained from Bio-Rad Laboratories, Hercules, CA; Dulbecco’s modified eagle’s medium (DMEM) for primary cell culture was obtained from Fisher Scientific. All other chemicals were obtained from Sigma, St. Louis, MO.

B2. Animals

New Zealand white rabbits (weight: 4-5 lbs) were purchased from RSI Biotechnology, Clemmons, NC and euthanized by sodium pentobarbital overdose (100 mg/kg), as approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University. The animals were housed in the animal facility administered by the Division of Animal Resources, Virginia Commonwealth University. All procedures were conducted in accordance with the Institutional Animal Care and Use Committee of the Virginia Commonwealth University.

B3. Preparation of dispersed smooth muscle cells.
Smooth muscle cells were isolated from the circular muscle layer of rabbit proximal stomach (fundus) by sequential enzymatic digestion, filtration, and centrifugation, as previously described. Briefly, smooth muscle strips were incubated for 30 min at 31°C in 15 ml of medium containing 120 mM NaCl, 4 mM KCl, 2.6 mM KH₂PO₄, 0.6 mM MgCl₂, 25 mM HEPES, 14 mM glucose, 2.1% (v/v) Eagle’s essential amino acid mixture, 0.1% collagenase (type II), and 0.1% soybean trypsin inhibitor. Partly digested tissues were washed with 50 ml of enzyme-free medium and muscle cells allowed to disperse spontaneously. The cells were harvested by filtration through 500μm Nitex and centrifuged twice at 350g for 10 min to eliminate cells and organelles. The experiments were done within 2-3 hours of isolation.

**B4- Preparation of cultured gastric smooth muscle cells**

Dispersed muscle cells isolated from the stomach were resuspended in DMEM containing penicillin (200 U/ml), streptomycin (200 μg/ml), gentamycin (100 μg/ml), amphotericin B (2.5 μg/ml) and 10% fetal bovine serum (DMEM-10). The muscle cells were plated at a concentration of 5 X 10⁵ cells/ml and incubated at 37°C in a 10% CO₂ incubator. DMEM-10 medium was replaced every three days for 2-3 weeks until confluence was attained. The muscle cells in confluent primary cultures were trypsinized (0.5 mg trypsin/ml), re-plated at a concentration of 2.5 X 10⁵ cells/ml and cultured under the same conditions. All experiments were done on cells in the first passage.

**B5. RT-PCR analysis of CBS and CSE.**

Specific primers were designed based on homologous sequences in human, rat, and mouse CBS and CSE. The primers for Rabbit CSE were Forward 5'ACA TTT CGC CAC GCA GGC CA-3' and, Reverse 5'CTC CCA GAG CCA AAG GCC GC-3' generating a fragment of
The primers for mouse CSE were Forward 5'GGG CAT CTG CAG GGA AAG GAA CCG-3' and Reverse 5'GCA GAT TGG TCC ACG CCC CT-3', generating a fragment of 851 bp. The primers for human CSE were Forward 5'TGG ATG GGG CTA AGT ACT GTT TGG C-3' and Reverse 5'CAG AGC CAA AGG GCG CTG GAA A-3', generating a fragment of 312 bp.

Cultured gastric muscle cells were treated with RNaqueous reagent (Ambion, Austin, TX) followed by an extraction with phenol:chloroform:isoamylalcohol (25:24:1). RNA (5 µl) was used to synthesize cDNA using Superscript II reverse transcriptase (Applied Biosystems, Foster, CA) with random hexanucleotide primers. Reversibly transcribed cDNA (5 µl) was amplified by PCR under standard conditions using the HotMaster Taq DNA polymerase kit (Epicentere Biotechnologies, Madison, WI) in a final volume of 50 µl containing 100 ng of each primer. The PCR products were separated by electrophoresis in 1.2% agarose gel in the presence of ethidium bromide, visualized by ultraviolet fluorescence, and recorded by a ChemiImager 4400 Fluorescence system.

**B6. Western blot analysis for CBS and CSE.**

Freshly dispersed or cultured smooth muscle cells were solubilized in Triton X-100-based lysis buffer plus protease and phosphatase inhibitors. After centrifugation of the lysates at 20,000 g for 10 min at 4°C, protein concentrations of the supernatant were determined with the DC Protein Assay kit from Bio-Rad (Hercules, CA). Proteins (50 µg) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Immobilon-FL, Millipore, Billerica, MA). The blots were incubated for 12 h at 4°C with antibodies (1:1,000) to CBS and CSE and then for 1 h with secondary antibody conjugated with horseradish peroxidase. The protein bands were visualized by enhanced chemi-luminescence.
CSE-targeted 21 nucleotide siRNA duplex was purchased from Ambion (Austin, TX, USA). Negative control siRNA, a 21 nucleotide RNA duplex with no sequence homology with all known genes, was also purchased from Ambion. Transfection of siRNA into cultured smooth muscle cells from rabbit gastric was achieved using the lipofectamine 2000 transfection reagent from Invitrogen. Briefly, cells were plated overnight to form 80-90% confluent monolayers. CSE siRNA at 40 nM and the transfection reagent complex were added to cells in serum-free medium for 24 h. As a control, negative siRNA was used to transfect rabbit gastric cells.

B8. Measurement of adenylyl cyclase activity

Adenylyl cyclase activity was measured by the formation of the second messenger cAMP in response to the exogenous donor of H$_2$S, NaHS and the endogenous activator of H$_2$S, L-Cysteine. Radioimmunoassay using radiolabeled [$^{125}$I] cAMP was performed to measure the level of cAMP production. Forsoklin, which is an activator of adenylyl cyclase (AC) is used as a positive control. The levels of cAMP in response to the NaHS and L-Cysteine are compared to the cAMP levels induced by forsoklin. One milliliter of cell suspension contains approximately 3 x 10$^6$ cells/ml. The experiments were performed after the cells were treated with NaHS (1mM) and L-Cysteine (10mM). Another set of cells were treated with 10µM of forskolin. Following treatment the reaction was terminated with cold 6% trichloroacetic acid (v/v) and vortexed vigorously. The mixture was then centrifuged and the supernatant was collected and were extracted three times with water-saturated diethyl ether to remove the trichloroacetic acid and then lyophilized and frozen at -20°C. Before radioimmunoassay, the samples were reconstituted in 500 µl of 50mM sodium acetate (pH 6.2) and acetylated with triethylamine/acetic anhydride (2:1 v/v) for 30 min. Cyclic AMP was measured in triplicates using 100 µl aliquots and the
RESULTS were analyzed and derived from a standard curve using Prism @ GraphPad program. The results were expressed as pmol of cAMP/mg protein.

**B9. Assay for Rho kinase activity.**

Rho kinase activity was determined in cell extracts by immunokinase assay as previously described.\(^{137}\) Freshly dispersed smooth muscle cells from rabbit stomach were treated the contractile agonist carbachol (0.1 µM) in the presence or absence of different concentrations of NaHS or L-Cysteine for 10 min and solubilized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml pepstatin A, and 10 µg/ml leupeptin. Equal amounts of protein extracts were incubated with Rho kinase-2 antibody plus protein A/G agarose overnight at 4°C. Immunoprecipitates were washed twice with a phosphorylation buffer containing 10 mM MgCl\(_2\) and 40 mM HEPES (pH 7.4) and then incubated for 5 min on ice with myelin basic protein (MBP) (1 mg/ml) as a substrate for Rho kinase activity. The kinase reaction was initiated by the addition of 10 µCi of \([^{32}\text{P}]\text{ATP}\) (3,000 Ci/mmol) and 20 µM ATP, followed by an incubation for 10 min at 37°C. \(^{32}\text{P}\)-labeled myelin basic protein was absorbed onto phosphocellulose disks, and repeated washings with 75 mM phosphoric acid removed unbound \([^{32}\text{P}]\text{ATP}\). The extent of phosphorylation was determined from the radioactivity on phosphocellulose discs by liquid scintillation.

**B10. Measurement of contraction and relaxation in muscle strips.**

Circular muscle Strips from the proximal stomach of rabbit were collected and rinsed immediately in Kreb’s solution containing 118 mM NaCl, 4.8 mM KCl, 1mM MgSO\(_4\), 1.15 mM NaH\(_2\)PO\(_4\), 15 mM NaHCO\(_3\), 10.5 mM Glucose and 2.5 mM CaCl\(_2\). Muscle strips were cut along
the circular axis and tied at each end with silk thread and mounted vertically in 5 ml tissue bath containing oxygenated (95% O₂/5% CO₂) Krebs solution at a pH of 7.4 at 37°C. The tissues were mounted between glass rod and an isometric transducers (Grass Technologies) connected to a computer recording system (Polyview). Preparations were allowed to equilibrate for 1 h at resting tension (2 g) before initiation of experiments and bath buffer solution was changed every 15 minutes during equilibration. To measure NaHS or L-cysteine induced relaxation, the strips were precontracted with 10 μM CCh, and after obtaining stable sustained contraction different concentrations of L-cysteine or NaHS were cumulatively added. In a separate study 10 μM glibenclamide, a K<sub>ATP</sub> channel blocker was added to the organ bath for 15 min before CCh or NaHS or L-cysteine treatment. At the end of each experiment, the strips were blotted dry and weighed (tissue wet weight). Contractile activity of muscle strips from stomach was calculated as maximum force generated in response to CCh and relaxation was calculated as percent decrease in maximum contraction.

B11. Measurement of contraction and relaxation in freshly isolated muscle cells.

Contraction in freshly dispersed gastric smooth muscle cells was determined by scanning micrometry as previously described (22-24). Freshly isolated muscle cells (0.4 ml) containing (10<sup>4</sup>/cell ml) from proximal stomach were preincubated with different concentrations of L-cysteine, NaHS and then 10μM CCh was added for 10 min. The reaction was terminated with 1% acrolein. The same experiments were repeated in cells preincubated for 15 min with a K<sub>ATP</sub> channel inhibitor 10 μM glibenclamide. The resting cell length was determined in control experiments in which muscle cells were incubated with 100 μl of 0.1% bovine serum albumin in the absence of CCh. The mean cell length of 50 muscle cells was measured by scanning.
Contraction in response to CCh was expressed as decrease in mean cell length from control cell length and relaxation was measured as percent decrease in contractile response in the presence of L-cysteine and NaHS.

**B12. Statistical Analysis**

The results were expressed as means ±S.E.M of n experiments and analyzed for statistical significance using Student t-test for paired and unpaired values. Each experiment was performed on cells and tissues obtained from different animals. Difference of responses among different groups were tested and confirmed for significance using Fisher’s protected least significance difference test. The results were analyzed using GraphPad software, San Diego, CA. A probability of P< 0.05 was considered significance.
RESULTS

C1. Expression of CSE enzyme in smooth muscle cells from stomach and colon.

To examine the role of endogenous H$_2$S in the regulation of muscle function, expression of the two main enzymes involved in the synthesis of H$_2$S was determined by RT-PCR and western blot. mRNA expression was determined in cultured muscle cells to avoid amplification from cell other than muscle cells. Previous studies have determined the purity of cultured muscle cells with smooth muscle-specific γ-actin and absence of interstitial cells of Cajal and endothelial cells in the primary cultures after first passage$^{138}$ Expression of mRNA for CSE, but not CBS was detected in muscle cells from stomach and colon. A PCR product of the expected size was obtained with CSE specific primers using RNA isolated from cultured muscle cells derived from the stomach and colon of rabbit (560 bp), mouse (851 bp) and human (312 bp) (Fig. 1A). Under similar conditions, there was no detectable PCR product with CBS-specific primers using RNA from cultured muscle cells of the stomach and colon of rabbit, mouse and human. However, a PCR product of expected size was obtained using CBS-specific primers with RNA isolated from mouse STC-1 cells (data not shown).

Western blot analysis using specific antibody to CSE demonstrated the presence of CSE protein (66 kDa) in lysates derived from muscle cells of the stomach and colon (Fig. 1B). Under similar conditions, there was no detectable expression of CBS protein with the lysates derived from dispersed muscle cells of the stomach and colon of rabbit, mouse and human. However, expression of CBS protein was obtained using CBS-specific antibody with lysates prepared from mouse STC-1 cells (data not shown). These results suggest that muscle cells selectively express CSE and this is consistent with the tissue-specific expression of CSE and CBS.$^{139}$
We next examined the role of \( \text{H}_2\text{S} \) in the regulation of muscle function by isometric contraction and scanning micrometry. Isometric contraction was measured by organ bath in muscle strips and by scanning micrometry in freshly dispersed muscle cells. Muscle strips from the proximal stomach were allowed to equilibrate to a passive tension 2g for 1hr before experiments were conducted. Sustained peak contraction in response to CCh was defined as tone above basal. As shown previously\(^{140}\), carbachol (CCh, 10 \( \mu \text{M} \)) induced a sustained contraction of 12 g above basal tension. Addition of L-cysteine caused inhibition of CCh-induced contraction (i.e. relaxation) in a concentration-dependent fashion (Fig. 2). Relaxation responses were 5\( \pm \)3 % at 1 mM L-cysteine (NS), 21\( \pm \)3% at 10 mM L-cysteine (p<0.01), and 55\( \pm \)5 % at 100 mM L-cysteine (p<0.01). Previous studies\(^{141}\) in vascular and visceral muscle have shown that relaxation in response to \( \text{H}_2\text{S} \) was mediated by activation of plasma membrane \( \text{K}_{\text{ATP}} \) channels and hyperpolarization. The notion was examined using \( \text{K}_{\text{ATP}} \) channel inhibitor glibenclamide. Incubation of muscle strips with glibenclamide (10\( \mu \text{M} \)) did not affect the relaxation in response to L-cysteine: 18\( \pm \)4% at 1 mM L-cysteine (p<0.01), 28\( \pm \)4% at 10 mM L-cysteine (p<0.01), and 68\( \pm \)5% at 100 mM L-cysteine (p<0.001) (Fig 2). The results suggest that relaxation of sustained contraction by L-cysteine was not due to activation of \( \text{K}_{\text{ATP}} \) channels. Addition of \( \text{H}_2\text{S} \) donor, NaHS also caused inhibition of CCh-induced contraction in a concentration-dependent fashion (Fig. 3). Relaxation responses were 52\( \pm \)4 % at 1 mM NaHS (p<0.001), 97\( \pm \)8% at 10 mM NaHS (p<0.001), and 100\( \pm \)9% at 100 mM NaHS (p<0.001). As in response to L-cysteine, relaxation in response to NaHS was not affected by incubation of muscle strips with glibenclamide (Fig. 3): 68\( \pm \)5% at 1 mM NaHS (p<0.001), 97\( \pm \)10% at 10 mM NaHS
C3. Inhibition of contraction by H$_2$S in isolated muscle cells.

Relaxation in response to L-cysteine and NaHS in innervated muscle strips could be due to release of transmitters from the enteric neurons, which in turn, modulate the intrinsic electrical and mechanical activity of the gastrointestinal smooth muscle. To avoid the confounding effects of neural activation by H$_2$S, we examined the effect of L-cysteine and NaHS on CCh-induced muscle contraction in freshly dispersed muscle cells by scanning micrometry. As in muscle strips, contractile agonist induce biphasic contraction in isolated muscle cells: a rapid contraction within 30 s followed by a sustained contraction for 20 min. To examine the effect of L-cysteine or NaHS on sustained contraction, muscle cells were treated with different concentrations of CCh (0.1 nM to 10 μM) in the presence or absence of L-cysteine (10 mM) or NaHS (1 mM) for 10 min. CCh caused a concentration-dependent contraction in dispersed muscle cells with an EC$_{50}$ of 12 nM. In the presence of L-cysteine or NaHS the contractile response to CCh was shifted to the right suggesting inhibition of contraction with EC$_{50}$ values of 25 nM and 79 nM, respectively (Fig. 4). Addition of L-cysteine caused inhibition of CCh-induced contraction (i.e. relaxation) in a concentration-dependent fashion (Fig. 5). Relaxation responses were 35±5% at 1 mM L-cysteine (p<0.001), 58±6% at 10 mM L-cysteine (p<0.001), and 79±8% at 100 mM L-cysteine (p<0.001). Addition of NaHS also caused relaxation in a concentration-dependent fashion (Fig. 6). Relaxation responses were 50±5 % at 1 mM NaHS (p<0.001), 65± 7% at 10 mM NaHS (p<0.001), and 83±6% at 100 mM NaHS (p<0.001) (Fig. 6). The effect of 10 mM L-cysteine (39±3% vs 36±4% relaxation ) or 1 mM NaHS (48±5% vs. 47±6% relaxation) on CCh-induced contraction was not affected in the presence of
Control studies showed that relaxation (52±7% relaxation) in response to levomakalim, a potassium channel activator was inhibited by glibenclamide (8±4% relaxation) (Fig. 8). The results suggest that both endogenous (via activation of CSE by L-cysteine) and exogenous H$_2$S inhibit muscle contraction. The results also suggest that the inhibitory effect of H$_2$S is independent of K$_{ATP}$ channel activation.

**C4. Mechanism of action of H$_2$S to mediate muscle relaxation**

Previous studies have shown that muscle relaxation in response to inhibitory transmitters such as VIP or NO was mediated by generation of cAMP and cGMP, and activation of cAMP-dependent kinase (PKA) and cGMP-dependent protein kinase (PKG), respectively. Selective inhibitors of PKA (H-89) or PKG (Rp-cGMPS) were used to examine the involvement of PKA and PKG in L-cysteine- and NaHS-induced relaxation. Relaxation in response to 10 mM L-cysteine (48±5% relaxation) was not affected by 1 µM H-89 (51±6% relaxation) or 1 µM Rp-cGMPS (42±5% relaxation) (Fig. 9). Similarly, relaxation in response to 1 mM NaHS (59±4% relaxation) was not affected by 1 µM H-89 (55±5% relaxation) or 1 µM Rp-cGMPS (51±4% relaxation) (Fig. 9). Control studies showed that relaxation in response to NO donor, S-nitrosoglutathione (GSNO, 10 µM) which generates cGMP and activates PKG was inhibited by Rp-cGMPS, but not by H-89 (61±8% relaxation with GSNO, 12±4% relaxation with GSNO and Rp-cGMPS, and 52±6% relaxation with GSNO and H-89) (Fig 10). Relaxation in response to forskolin, which generates cAMP and activates PKA was inhibited by H-89, but not by Rp-cGMPS (65±4% relaxation with forskolin, 12±4% relaxation with forskolin and H-89, and 58±5% relaxation with forskolin and Rp-cGMPS) (Fig. 10). These results suggest that relaxation by H$_2$S was independent of PKA and PKG activation.
C4a. Effect of \( \text{H}_2\text{S} \) on cAMP levels in isolated muscle cells from the stomach

Lack of effect of PKA inhibitor on muscle relaxation in response to L-cysteine and NaHS was corroborated by measurements of cAMP. Muscle cells were treated with L-cysteine (10 mM), NaHS (1 mM) or forskolin (10\( \mu \)M) for 5 min in the presence of IBMX (10 \( \mu \)M) and cAMP levels are measured by radioimmuno assay. Treatment of cells with L-cysteine (2.1±0.35 pmol/mg protein) or NaHS (2.6±0.4 pmol/mg protein) did not change the levels of cAMP compared to basal cAMP levels (1.8±0.25 pmol/mg protein) (Fig. 11). In contrast, forskolin caused a significant increase (17.6±2.1 pmol/mg protein, \( p<0.001 \)) in cAMP levels above basal levels (Fig. 11). These results suggest that \( \text{H}_2\text{S} \) does not stimulate adenylyl cyclase activity and increase the cAMP levels in the gastric muscle cells; but induce relaxation through other pathways that inhibit contraction.

C4b. Inhibition of Rho kinase activity by \( \text{H}_2\text{S} \).

Previous studies in gastric muscle have shown sustained contraction in response to CCh is mediated by activation of RhoA/Rho kinase pathways involving phosphorylation of MYPT1, the regulatory subunit of MLC phosphatase, via Rho kinase leading to inhibition of MLC phosphatase and increase in MLC\(_{20}\) phosphorylation, a prerequisite for contraction. As shown previously, sustained contraction (31±3\% decrease in cell length) in response to CCh was inhibited by the selective Rho kinase inhibitor, Y27632 (1 \( \mu \)M) (4±4\% decrease in cell length)\(^{140} \) (Fig. 12). We next examined the hypothesis that inhibition of contraction by \( \text{H}_2\text{S} \) is due to inhibition of Rho kinase leading to activation of MLC phosphatase and MLC\(_{20}\) dephosphorylation, a prerequisite for relaxation.
As shown previously, treatment of cells with 0.1 μM CCh for 10 min caused a significant increase in Rho kinase activity above basal levels (268±12% increase, p<0.001) (Fig. 13). Addition of L-cysteine (10 mM) caused significant inhibition of CCh-stimulated Rho kinase activity (62±4% inhibition, p<0.001). Similarly, addition of NaHS (1 mM) caused significant inhibition of CCh-stimulated Rho kinase activity (76±6% inhibition, p<0.001) (Fig. 13). These results suggest that relaxation of sustained contraction by H₂S was due to inhibition of Rho kinase activity.

**C4c. Inhibition of Rho kinase activity by L-cysteine is mediated via CSE/ H₂S**

The involvement of endogenous H₂S generation in the inhibition of Rho kinase by L-cysteine was examined by transfection of cultured muscle cells with CSE specific siRNA. Suppression of CSE was verified by western blot (Fig. 14). CCh induced significant increase (238±15% increase above basal level) in Rho kinase activity in cultured muscle cells. Addition of L-cysteine (10 mM) caused significant inhibition of CCh-stimulated Rho kinase activity (85±8% inhibition) in cells transfected with control siRNA but not in cells transfected with CSE-specific siRNA (19±9% inhibition) (Fig. 14). These results provide evidence for the involvement of CSE, probably via generation of H₂S, in the inhibition of Rho kinase activity.

**In summary,**

1. CSE, but not CBS is expressed in gastric smooth muscle cells.

2. L-cysteine, an activator of CSE, and H₂S donor NaHS caused relaxation of sustained contraction in a concentration-dependent fashion in muscle strips. Relaxation by L-cysteine and NaHS was not affected by the Kₐ₅₉ channel blocker, glibenclamide.
Relaxation by L-cysteine and NaHS was not affected by the K\textsubscript{ATP} channel blocker, glibenclamide, PKA inhibitor, H-89 or PKG inhibitor, Rp-cGMPS. L-cysteine and NaHS had no effect on cAMP levels.

4. L-cysteine and NaHS caused inhibition of CCh-stimulated Rho kinase activity. Previous studies have shown that sustained contraction by CCh was inhibited by the selective Rho kinase inhibitor, Y27632 (1 µM).

5. Inhibition of Rho kinase activity by L-cysteine was blocked in cells transfected with CSE specific siRNA.
Figure 1. Expression of CSE in smooth muscle cells. (A) CSE expression was identified in cultured muscle cells from stomach and colon of rabbit, mouse and human by RT-PCR. A PCR product of the expected size was obtained with CSE specific primers using RNA isolated from cultured muscle cells derived from stomach and colon of rabbit (560 bp), mouse (851 bp) and human (312 bp). Under similar conditions, there was no detectable PCR product with CBS-specific primers from cultured muscle cells of stomach and colon of rabbit, mouse and human. (B) Western blot analysis using specific antibody to CSE (1:1000) demonstrated the presence of CSE protein (66 kDa) in lysates derived from muscle cells of stomach and colon of rabbit, mouse and human. Under similar conditions, there was no detectable expression of CBS protein with the lysates derived from dispersed muscle cells of stomach and colon of rabbit, mouse and human.
Figure 1-A: Expression of mRNA

- Rabbit: Stomach (560 bp), Colon
- Human: Stomach (312 bp), Colon
- Mouse: Stomach, Colon (851 bp)

Figure 1-B: CSE protein expression

Mouse, Rabbit, Human

66 kDa
Figure 2. Effect of L-cysteine on carbachol-induced contraction in gastric muscle strips. Muscle strips from rabbit proximal stomach were allowed to equilibrate to a passive tension of 2g for 30 min and then treated with carbachol (CCh, 10 µM). The increase in tension (increase in the amplitude) in response to CCh was calculated as contraction. After the muscle attained sustained contraction, different concentrations of L-cysteine was added in the presence or absence of glibenclamide (10 µM). Inhibition of contraction was calculated as relaxation and expressed as percent inhibition of maximum tension. Values are means ± SEM of 4 experiments. ** p<0.001 significant inhibition of CCh-induced contraction.
Figure 2
Figure 3. Effect of NaHS on carbachol-induced contraction in gastric muscle strips. Muscle strips from rabbit proximal stomach were allowed to equilibrate to a passive tension of 2g for 30 min and then treated with carbachol (CCh, 10 µM). The increase in tension (increase in the amplitude) in response to CCh was calculated as contraction. After the muscle attained sustained contraction, different concentrations of NaHS was added in the presence or absence of glibenclamide (10 µM). Inhibition of contraction was calculated as relaxation and expressed as percent inhibition of maximum tension. Values are means ± SEM of 4 experiments. ** p<0.001 significant inhibition of CCh-induced contraction.
Figure 3

![Graph showing relaxation (%) for NaHS and NaHS + Glibenclamide at concentrations of 1, 10, and 100 mM.]
Figure 4. Effect of L-cysteine and NaHS on carbachol-induced contraction in isolated muscle cells of stomach. Muscle cells isolated from proximal stomach were treated with different concentrations of CCh (0.1nM to 10 µM) for 10 min. In some experiments cells were pretreated for 10 min with 10mM L-cysteine or 1mM NaHS and then treated with CCh. Muscle cell length was measured by scanning micrometry. Contraction by CCh was calculated as decrease in muscle length from control cell length of 104±5 µm. Values are means ± SEM of 7 experiments.
Figure 4

![Graph showing contraction (% decrease in cell length) vs. Carbachol (log M) for different treatments: CCh, CCh + L-cys, CCh + NaHS. The graph illustrates the effect of Carbachol in various concentrations on the cells, with error bars indicating variability.]
Figure 5. Effect of L-cysteine on carbachol-induced contraction in isolated muscle cells of stomach. Muscle cells isolated from proximal stomach were treated with CCh (0.1 µM) for 10 min to induce sustained contraction. In some experiments cells were pretreated with different concentrations of L-cysteine for 10 min and then treated with CCh for 10 min. Muscle cell length was measured by scanning micrometry. Contraction by CCh was calculated as decrease in muscle length from control cell length (30±3% decrease in cell length from the basal cell length of 109±5 µm). Relaxation by L-cysteine was expressed as percent decrease in CCh-induced contraction. Values are means ± SEM of 7 experiments. ** p<0.001, significant inhibition of CCh-induced contraction.
Figure 6. Effect of NaHS on carbachol-induced contraction in isolated muscle cells of stomach. Muscle cells isolated from proximal stomach were treated with CCh (0.1 µM) for 10 min to induce sustained contraction. In some experiments cells were pretreated with different concentrations of NaHS for 10 min and then treated with CCh for 10 min. Muscle cell length was measured by scanning micrometry. Contraction by CCh was calculated as decrease in muscle length from control cell length (30±3% decrease in cell length from the basal cell length of 109±5 µm). Relaxation by NaHS was expressed as percent decrease in CCh-induced contraction. Values are means ± SEM of 7 experiments. ** p<0.001, significant inhibition of CCh-induced contraction.
Figure 6
Figure 7. Effect of glibenclamide on relaxation induced by L-cysteine and NaHS in isolated muscle cells of stomach. Muscle cells isolated from proximal stomach were treated with CCh (0.1 µM) for 10 min to induce sustained contraction. In some experiments cells were pretreated with L-cysteine (10 mM) or NaHS (1 mM) in the presence or absence of glibenclamide (10 µM) for 10 min and then treated with CCh for 10 min. Muscle cell length was measured by scanning micrometry. Contraction by CCh was calculated as decrease in muscle length from control cell length (29±4% decrease in cell length from the basal cell length 104±5 μm). Relaxation in response to L-cysteine or NaHS was expressed as percent decrease in contraction. Values are means ± SEM of 7 experiments. ** p< 0.01, significant inhibition of CCh-induced muscle contraction.
Figure 7

![Graph showing relaxation percentage for L-Cys (10 mM) and Gliben in comparison to NaHS (1 mM) and Gliben. The graph indicates significant differences marked with **.](image)
Figure 8. Effect of glibenclamide on relaxation induced by levocromakalin in isolated muscle cells of stomach. Muscle cells isolated from proximal stomach were treated with CCh (0.1 µM) for 10 min to induce sustained contraction. In some experiments cells were pretreated with levocromakalin (10 µM) in the presence or absence of glibenclamide (10 µM) for 10 min and then treated with CCh for 10 min. Muscle cell length was measured by scanning micrometry. Contraction by CCh was calculated as decrease in muscle length from control cell length (29±4% decrease in cell length from the basal cell length 104±5 µm). Relaxation in response to levocromakalin was expressed as percent decrease in contraction. Values are means ± SEM of 4 experiments. ** p< 0.001, significant inhibition of CCh-induced muscle contraction.
Figure 8
**Figure 9. Effect of H-89 and Rp-cGMPS on relaxation induced by L-cysteine and NaHS in isolated muscle cells of stomach.** Muscle cells isolated from proximal stomach were treated with CCh (0.1 µM) for 10 min to induce sustained contraction. In some experiments cells were pretreated with L-cysteine (10 mM) or NaHS (1 mM) in the presence or absence of PKA inhibitor, H-89 (1 µM) or PKG inhibitor, Rp, cGMPS (1 µM) for 10 min and then treated with CCh for 10 min. Muscle cell length was measured by scanning micrometry. Contraction by CCh was calculated as decrease in muscle length from control cell length (29±4% decrease in cell length from the basal cell length 104±5 µm). Relaxation in response to L-cysteine or NaHS was expressed as percent decrease in contraction. Values are means ± SEM of 7 experiments. ** p< 0.001, significant inhibition of CCh-induced muscle contraction.
Figure 9
Figure 10. Effect of H-89 and Rp-cGMPS on relaxation induced by forskolin and S-nitrosothiol in isolated muscle cells of stomach. Muscle cells isolated from proximal stomach were treated with CCh (0.1 µM) for 10 min to induce sustained contraction. In some experiments cells were pretreated with GSNO (10 µM) in the presence or absence of Rp-cGMPS (1 µM) or forskolin (10 µM) in the presence or absence of H-89 (1 µM) for 10 min and then treated with CCh for 10 min. Muscle cell length was measured by scanning micrometry. Contraction by CCh was calculated as decrease in muscle length from control cell length (29±4% decrease in cell length from the basal cell length 104±5 μm). Relaxation in response to GSNO or forskolin was expressed as percent decrease in contraction. Values are means ± SEM of 4 experiments. ** p< 0.001, significant inhibition of CCh-induced muscle contraction.
Figure 10

**Graph 1:**
- Relaxation (%)
- Fsk
- H89
- RpG

**Graph 2:**
- Relaxation (%)
- GSNO
- H89
- RpG
Figure 11: Effect of L-cysteine and NaHS on adenylyl cyclase activity in isolated muscle cells of stomach. Muscle cells isolated from proximal stomach were treated with NaHS (1 mM) and L-Cysteine (10 mM), or forskolin (10 µM) for 5 min. Stimulation of adenylyl cyclase activity was measured as increase in cAMP levels above basal levels. cAMP levels were measured by radioimmunoassay. Results are expressed as pmol/mg protein. ** p<0.001 significant increase in cAMP levels.
Figure 12. Effect of Rho kinase inhibitor in CCh-induced sustained contraction. Muscle cells isolated from proximal stomach were treated with CCh (0.1 µM) in the presence or absence of the Rho kinase inhibitor Y27632 (1 µM) for 10 min to induce sustained contraction. Muscle cell length was measured by scanning micrometry. Contraction by CCh was calculated as decrease in muscle length from control cell length (31±3% decrease in cell length from the basal cell length 101±6 µm). Values are means ± SEM of 4 experiments. ** p< 0.01, significant inhibition of CCh-induced muscle contraction.
Figure 12

![Graph showing contraction (% decrease in cell length) comparison between CCh and CCh + Y27632. The graph indicates a significant decrease in contraction when Y27632 is added to CCh.]
Figure 13. Effect of L-cysteine and NaHS on carbachol-stimulated Rho kinase activity in isolated muscle cells of stomach. Muscle cells isolated from proximal stomach were treated with CCh for 10 min. In some experiments cells were pretreated with L-cysteine (10 mM) or NaHS (1 mM) for 10 min and then treated with CCh for 10 min. Rho kinase activity was measured by immunokinase assay using $[^{32}P]ATP$. Results are expressed as cpm/mg protein. Values are means ±SEM of 4-6 experiments. **p<0.001 significant inhibition of CCh-stimulated Rho kinase activity.
Figure 13

![Graph showing Rho kinase activity (cpm/mg protein) for Basal, CCh, L-Cys, and NaHS conditions.](image)
Figure 14. Effect of CSE siRNA on L-cysteine-induced inhibition of Rho kinase activity. Cultured gastric muscle cells were transfected with control siRNA or CSE-specific siRNA for 48 h. Expression of CSE was measured by western blot (inset). Cells were treated with CCh (0.1 µM) for 10 min. In some experiments the transfected cells were pretreated for 10 min with L-cysteine (10 mM) and then treated with CCh for 10 min. Rho kinase activity was measured by immunokinase assay using $[^{32}\text{P}]\text{ATP}$. Results are expressed as cpm/mg protein. Values are means±SEM of 4-6 experiments. **p< 0.001, significant inhibition of CCh-stimulated Rho kinase activity by L-cysteine.
Figure 14
D. DISCUSSION

Though the existence of H$_2$S in biological tissues has been known for over 300 years, it is the most recently appreciated of the gasotransmitters as a physiologic messenger molecule. Regarded as a third gasotransmitter, H$_2$S is receiving increasing interest, as much as NO and CO has received previously, to understand its physiological functions.$^{142}$ Using exogenous H$_2$S donors or activators of endogenous enzymes such as CSE and CBS that generate H$_2$S, CSE siRNA and CSE$^{-/-}$ mice, regulation of several functions by H$_2$S has been demonstrated and these include cardiovascular, gastrointestinal and central nervous system, and energy metabolism. Inhibitors of these enzymes have been employed to characterize influences of H$_2$S in various organs in mammals. Definitive evidence that H$_2$S is a physiologic regulator came with the knockout mice with targeted deletion of CSE and CBS.

In the gastrointestinal (GI) tract, H$_2$S synthesizing enzymes are expressed by enteric neurons, interstitial cells of Cajal and in the epithelium.$^{143}$ In addition, the gut microbiota produces H$_2$S which leads to luminal concentrations of H$_2$S of up to 1 mM.$^{144}$ Transcripts encoding both CSE and CBS have been identified in different cell types in the GI system. H$_2$S is produced by activation of these enzymes but no evidence indicates the physiological mechanisms of activation of the H$_2$S synthesizing enzymes. Recently, several studies reported actions of H$_2$S on various GI functions. These functions include inflammatory and anti-inflammatory, nociceptive and anti-nociceptive actions, and regulation of gastric mucosal blood flow, secretion and GI motility.$^{145}$ Currently, the most advanced efforts to develop therapeutic agents involve the combination of H$_2$S donors with non-steroidal anti-inflammatory drugs (NSAIDs). The H$_2$S releasing moiety provides cytoprotection to gastric mucosa normally adversely affected by
S and inhibition of prostaglandin synthesis may afford synergistic anti-inflammatory influences.

The important findings of our study are summarized as follows: i) transcripts of the H$_2$S-synthesizing enzymes CSE, but not CBS have been detected in isolated muscle cells, where it is responsible for H$_2$S production; ii) endogenous activation of CSE by L-cysteine inhibited agonist-induced Rho kinase activity and muscle contraction; iii) suppression of CSE expression blocked the effect of L-cysteine on Rho kinase activity providing strong evidence that H$_2$S may be responsible for L-cysteine-induced inhibitory effects; iv) exogenous H$_2$S donor, NaHS inhibited agonist-induced Rho kinase activity and muscle contraction, v) the effect of L-cysteine and NaHS was not affected by blockade of K$_{ATP}$ channel with glibenclamide, PKA with H-89 or PKG with Rp-cGMP. These studies suggest that a distinct PKA/PKG-independent mechanism via inhibition of Rho kinase activity mediates muscle relaxation by H$_2$S.

Recent studies in vascular and visceral smooth muscle have clearly established the H$_2$S as a mediator of relaxation. Expression of CSE is down regulated in hypertensive animal models. CSE$^+$ mice are hypertensive and exhibit reduced endothelium-dependent vasorelaxation; these effects are associated with decrease in H$_2$S generation in these mice. Exogenous H$_2$S administration reduced blood pressure, whereas inhibition of CSE activity with DL-propargylglycine increased blood pressure in rats. Extensive experiments on vascular tissues strongly suggest that H$_2$S-induced vasorelaxation is mainly caused by K$_{ATP}$ channel openings. This notion is largely based on the ability of glibenclamide, a K$_{ATP}$ channel antagonist, to block the vasorelaxant effects of H$_2$S. Systemic administration of different H$_2$S donors inhibits visceral nociception by opening K$_{ATP}$ channels. The activation of K$_{ATP}$ channels in the peripheral nociceptive system has been seen to be involved in the modulation of nociception. Additional
channels, Ca\(^{2+}\)-dependent K\(^+\) channels and ill-defined endothelium-dependent mechanisms appear to play a minor role in H\(_2\)S induced vasodilation. Generalization of these inhibitory mechanisms to other smooth muscle is problematic. The mechanism of inhibition of contraction by H\(_2\)S appears to be species- and tissue-specific, and varies with activation of the muscle. Inhibition of contraction by NaHS in urinary bladder was abolished by capsaicin suggesting the involvement of TRPV1 receptors. Glibenclamide insensitive effect of NaHS was also observed in mouse aorta, mouse bronchial rings, and mouse gastric fundus and guinea pig ileum.\(^{149}\)\(^{150}\) However, inhibition of spontaneous contractions by NaHS in human and rat colon were affected by glibenclamide and apamin. In these nerve-muscle preparation, H\(_2\)S may also affect enteric neurons to regulate smooth muscle function. A direct effect on muscle was implicated in distal colon of mouse and human since the Na\(^+\) channel blocker tetrodotoxin had no effect on the relaxation. However, an effect on interstitial cells of Cajal would also influence the function of smooth muscle in these preparations.

Although, exogenous NaHS has potent inhibitory effect on muscle contraction both in muscle strip preparations and isolated muscle cells, it was demonstrated that luminal application of NaHS was less effective than in isolated strips.\(^ {151}\) This could be due to effective barrier function of mucosa to limit the diffusion of H\(_2\)S to the muscle layers and efficient metabolism of H\(_2\)S to thiosulfate and sulphate by colonic mucosa. In humans and mice, H\(_2\)S can be produced upto 1mM range in the GI tract and efficient oxidation process by sulphide quinine oxidoreductase, sulphur dioxygenase and rhodanese would offer protection against high local concentrations.\(^ {152}\) Damage to this protective mucosal barrier function, as in the ulcerative colitis, may lead to increased access of H\(_2\)S to the inner muscle layers of GI tract.
Use of innervated muscle strips and isolated muscle strips limits the interpretation about the precise site of H_{2}S synthesis and its action. In the present study, using dispersed muscle cells and using activator of CSE and H_{2}S donor, we showed that H_{2}S inhibits CCh-induced sustained contraction. Evidence for the activation of CSE in mediating the effect of L-cysteine was based on selective expression of CSE in muscle cells. These results also strongly suggest that endogenous H_{2}S may be responsible for the L-cysteine-induced relaxation seen in our experiments. We carried out further experiments in dispersed muscle cells to investigate the mechanism of action of H_{2}S to mediate relaxation of sustained contraction. Our studies demonstrated that the inhibition of contraction is mediated via inhibition of CCh-induced Rho kinase activity. Evidence for this conclusion was based on inhibition of CCh-induce Rho kinase activity by both endogenous and exogenous H_{2}S and blockade of sustained contraction by Y27632, a selective Rho kinase inhibitor. Further evidence was provided using CSE siRNA. Suppression of CSE expression blocked the inhibitory effect of L-cysteine on Rho kinase activity. Glibenclamide, a selective inhibitor of K_{ATP} channels and a well-characterized target of H_{2}S to mediate muscle relaxation, had no effect on inhibition of contraction by L-cysteine or NaHS.

Previous studies have demonstrated that relaxation of RhoA/Rho kinase-mediated contraction by PKA and PKG involves phosphorylation of RhoA at Ser^{188} and inhibition of its ability to activate Rho kinase, phosphorylation of MYPT1 at Ser^{695} and blockade of inhibitory phosphorylation at the adjacent Thr^{696} by Rho kinase, and/or phosphorylation of telokin at Ser^{13}, an endogenous MLC phosphatase stimulator.^{153, 154} We examined whether H_{2}S mediated inhibition of Rho kinase activity and contraction involves cAMP- and/or cGMP-dependent mechanisms. Studies also showed that L-cysteine or NaHS had no effect on cAMP levels and
the effect of H$_2$S on contraction was not affected in the presence of PKA or PKG inhibitors. Similarly, inhibition of contraction by NaHS in mouse distal colon strips was not affected by NO synthase inhibitor, L-NAME, sGC inhibitor, ODQ, or SQ22536, adenylyl cyclase inhibitor observed in mouse distal colon strips. These results suggest that the relaxation by H$_2$S was mediated via a distinct mechanism independent of cAMP/PKA and cGMP/PKG pathways. H$_2$S act via protein sulfhydration to regulate various physiological functions. Future studies involve measurement of S-sulfhydration of Rho kinase or upstream targets such RhoA in response to L-cysteine or NaHS. S-sulfhydration provides a possible mechanism by which H$_2$S alters the function of several proteins and this process appears to common posttranslational modification and analogous to S-nitrosylation of proteins by NO. In this process, a hydropersulfide (-SSH) moiety is generated by the addition of sulphur from H$_2$S to the ÏŠH groups of cysteine residues and this results in altered chemical and biological reactivity of proteins. Snyder and colleagues have shown loss of protein sulfhydration in CSE$^{-/}$ mice compared to proteins in control mice. The covalent modification of proteins by sulfhydration is a well characterized mechanism, but the physiological mechanism in the reversal of sulfhydration is not clear. Sulfhydration is reversed by reducing agents such as dithiothreitol.

In summary, our studies have demonstrated that both exogenous and endogenous H$_2$S inhibits agonist-induced sustained muscle contraction in the stomach, and the inhibitory effect is mediated via inhibition of Rho kinase activity, a key enzyme in the regulation of MLC phosphatase activity and sustained muscle contraction. Inhibition of Rho kinase activity and ensuing disinhibition of MLC phosphatase activity leads to MLC$_{20}$ dephosphorylation, a prerequisite for muscle relaxation (Fig 15). The extent to which endogenous H$_2$S contributes to
intestinal motility under physiological conditions, and the physiological mechanism that regulate CSE activity and H₂S generation awaits further research.
Figure 15: Scheme depicting the pathways involved in the regulation of MLC20 phosphorylation and muscle contraction in smooth muscle. MLC$_{20}$ Phosphorylation in response to contractile agonists is initiated by stimulation of Ca$^{2+}$ calmodulin-dependent MLC kinase and sustained by inhibition of MLC phosphatase activity via Rho kinase-dependent phosphorylation of MLC phosphatase regulatory subunit. H$_2$S inhibits Rho kinase activity leading to stimulation of MLC phosphatase activity, MLC$_{20}$ dephosphorylation and muscle relaxation.
Figure 15
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