MINI-REVIEW

Hydrogen Sulfide: An Agent of Stability at the Microbiome-Mucosa Interface

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ABSTRACT

A diverse range of effects of the intestinal microbiota on mucosal defence and injury has become increasingly clear over the past decade. Hydrogen sulfide (H₂S) has emerged as an important mediator of many physiological functions, including gastrointestinal mucosal defence and repair. Hydrogen sulfide is produced by gastrointestinal tract tissues and by bacteria residing within the gut, and can influence the function of a wide range of cells. The microbiota also appears to be an important target of hydrogen sulfide. H₂S donors can modify the gut microbiota and the gastrointestinal epithelium is a major site of oxidation of microbial-derived H₂S. When administered together with nonsteroidal anti-inflammatory drugs, H₂S can prevent some of the dysbiosis those drugs induce, possibly contributing to the observed prevention of gastrointestinal damage. Exogenous H₂S can also markedly reduce the severity of experimental colitis and plays important roles in modulating epithelial cell-mucus-bacterial interactions in the intestine, contributing to its ability to promote resolution of inflammation and repair of tissue injury. In this paper we review recent studies examining the roles of H₂S in mucosal defence, the possibility that H₂S can damage the GI epithelium, effects of H₂S on the gut microbiota and on mucus and biofilm interactions in the context of intestinal inflammation.

KEYWORDS

Intestine; Colitis; NSAID; Epithelium; Bacteria; Microbiota; Mucus; Biofilm; Inflammatory Bowel Disease
Introduction

Over the past 15 years, our understanding of the roles of the gaseous mediator H$_2$S has grown considerably. It is now clear that H$_2$S plays important roles in many physiological and pathophysiological processes (22,44). Our laboratories have been particularly interested in the ability of H$_2$S to act as a mediator of inflammation, homeostasis and repair in the gastrointestinal (GI) tract. H$_2$S is an important mediator of mucosal defence, affecting such basic processes as mucosal blood flow, bicarbonate and mucus secretion, and endothelial-leukocyte interactions (13,19,30,49). Important roles for H$_2$S have also been demonstrated in visceral pain (7,10), and in resolution of inflammatory disorders such as colitis (14,18,23,43).

These observations have provided the impetus for several groups to design and develop novel H$_2$S-releasing drugs that may be used to protect the GI tract, accelerate repair of GI damage and reduce inflammation in diseases like ulcerative colitis, Crohn’s disease and NSAID-induced gastroenteropathy (7,21,44).

The intestinal microbiome is a significant source of H$_2$S, some of which permeates across the intestinal epithelium (15,25,35), providing an energy source for epithelial and other lamina propria cells (17,29). Of course, diet can affect the amounts of H$_2$S production. Organic polysulfides contained in garlic, onions, cruciferous vegetables (e.g., cabbage, cauliflower, kale, broccoli, etc.) and durian fruit can directly release H$_2$S through interactions with protein thiols or intracellular thiols (e.g., glutathione). L-cysteine is the main precursor for mammalian H$_2$S production, and H$_2$S synthesis can be modulated by dietary supplementation or restriction of this amino acid, or of homocysteine (a precursor of L-cysteine).
Since there is a clear role for microbiota in the pathogenesis of many GI disorders, investigations began into the possible interactions of H$_2$S with luminal bacteria. Many questions have arisen from these investigations: Can H$_2$S damage the intestinal epithelium and/or promote GI cancer? Does H$_2$S affect mucus secretion and/or function? Does H$_2$S modulate the microbiota? Does H$_2$S affect the interactions between microbiota and the epithelium?

Several recent studies have provided evidence that exogenous and endogenous H$_2$S can significantly reduce the susceptibility of the GI mucosa to injury, and these effects appear to be produced, at least in part, through modulation of the enteric microbiota (4,5,30). Adverse, dysbiosis-inducing effects of some commonly used drugs appear to contribute significantly to the detrimental effects of those drugs in the GI tract, but can be markedly attenuated by co-administration of H$_2$S. Indeed, a novel class of nonsteroidal anti-inflammatory drugs (NSAIDs) has been developed to exploit these beneficial effects of H$_2$S in countering the damaging effects of conventional NSAIDs (7), which are among the most widely used drugs.

**Is Luminal H$_2$S Toxic to the Intestinal Epithelium?**

H$_2$S is produced by a wide range of enteric bacteria, primarily of the $\gamma$-Proteobacteria genera (readers are referred to a comprehensive review by Linden (25)). H$_2$S concentrations in the cecum and rectum can reach 40 $\mu$M, (36) and as much as 250 $\mu$M in the colon (1). There have been a wide range of studies using different types of transformed or non-transformed epithelial cells to determine their responsiveness to a range of concentrations of H$_2$S, including concentrations well above 250 $\mu$M (25). Not surprisingly, there are discrepant findings from these studies, with no clear and consistent evidence for toxic effects of H$_2$S on epithelial cell
integrity, and considerable variability in terms of the effects of H$_2$S on epithelial cell proliferation (25). Linden (25) noted that H$_2$S (250 µM) could increase DNA damage in colon cancer cells in vitro, but only when DNA repair was inhibited (1). Indeed, there are numerous studies demonstrating anti-proliferative and chemo-preventive effects of H$_2$S in vitro and in vivo (9,24,32,33).

There has been speculation of potential links between bacterially derived H$_2$S and inflammatory bowel disease (31). These may include genotoxic properties and the disruption of the mucus structure. In our laboratory, we have done extensive studies in which H$_2$S donors were administered to rodents or dogs for periods of up to 2 weeks, but have not observed any evidence of tissue injury. Even with twice-daily administration of a garlic-derived H$_2$S donor (diallyl disulfide; DADS) for 5 days at a dose as high as 60 mmol/kg, there was no macroscopically or histologically detectable damage to the gastrointestinal epithelium, nor any mucosal inflammation (5). On the contrary, with administration of H$_2$S in this fashion, the normal structure of mucus and microbiota was maintained (30). Benavides et al. (3) demonstrated that when they added DADS to a solution of glutathione, to mimic in vivo conditions, approximately 50% was rapidly converted to H$_2$S.

On the other hand, a recent study of pediatric Crohn’s disease provided compelling evidence for a role of H$_2$S in promoting damage in a subset of patients who had a genetic defect in mitochondrial oxidation of H$_2$S (31). Interestingly, those patients also had substantially increased numbers of H$_2$S-producing bacteria in their intestinal lumen (31). We observed a similar increase in H$_2$S-producing intestinal bacteria in rat studies of the exacerbation of NSAID-enteropathy by proton pump inhibitors, which significantly worsened NSAID-induced
enteropathy (42). Our microbiome analysis revealed that a major effect of both omeprazole and lansoprazole was to significantly increase the numbers of γ-Proteobacteria in the small intestine (4).

Several studies in recent years have clearly demonstrated that H$_2$S is an important metabolic fuel for the epithelial cells that line the GI tract (17,29). Colonic epithelial cells are particularly efficient in oxidizing H$_2$S, producing ATP in the process (17,29). The epithelium therefore functions both as a physical barrier against potentially harmful agents that might pass into the body from the lumen of the gut, as well as a metabolic barrier, oxidizing bacteria-derived H$_2$S (41). A defect in this metabolic barrier function is what was observed in the pediatric Crohn’s disease patients mentioned in the preceding paragraph (31), which would allow H$_2$S from luminal bacteria to gain access to the lamina propria.

**Protective and Reparative Effects of H$_2$S**

H$_2$S has been shown to exert protective effects against GI injury induced by ethanol, NSAIDs and ischemia-reperfusion, as well as promoting resolution of inflammation and repair of tissue damage (41). For example, H$_2$S donors have been shown to promote resolution of colitis in several animal models (14,18,23,43). Endogenous H$_2$S production is markedly elevated at sites of mucosal injury, contributing significantly to promotion of healing (40,43). Moreover, rates of oxidation of H$_2$S are specifically and substantially reduced at sites of epithelial/mucosal injury (14). On the other hand, inhibition of H$_2$S synthesis leads to increased susceptibility to mucosal injury, impairment of healing, increased pro-inflammatory cytokine expression,
reduced expression of COX-2 (and an associated reduction of PG synthesis) and increased mucosal granulocyte numbers (5,14,30,43).

The protective effects of H2S in the GI tract have also been demonstrated in numerous studies of novel H2S-releasing NSAIDs. These drugs have been shown to cause negligible GI damage despite markedly suppressing prostaglandin synthesis in a wide range of animal models (39,40). While NSAIDs impair healing of gastric ulcers, H2S-releasing NSAIDs significantly accelerate healing, and this is mimicked by administration of H2S donors (39,40).

**H2S, Mucus & Biofilms**

Intestinal bacteria are thought to contribute significantly to the pathogenesis of inflammatory bowel disease (IBD; ulcerative colitis and Crohn’s disease) (28), but clear evidence for clinical benefits of antibiotics or probiotics for treating these conditions is lacking. A “leaky” mucus layer that can permit bacterial invasion has been reported in humans with ulcerative colitis, as well as in murine models of colitis (20). A recent mouse study reported that antibiotics caused a thinning of the mucus layer by directly reducing mucus granule numbers, an effect that predisposed mice to further enteric infection with *Citrobacter rodentium* (48).

In nature, bacteria form biofilms that reduce their exposure to drugs and immune components (6). They are complex, multispecies bacterial colonies encapsulated in a self-secreted matrix of polysaccharides (10,16). There is evidence that microbiota living as biofilms promote gut homeostasis (27,37). We attempted to determine the changes in biofilms that occur during experimental colitis, and the impact of reduced endogenous H2S synthesis versus administering exogenous H2S (30). Fluorescent *in situ* hybridization (FISH) was used to examine
the organization of gut microbiota in health and during colitis in rats, and the role that H$_2$S might play in modulating biofilm formation and stability.

In both mice and rats, the microbiota is sandwiched between the sterile mucus layer and the luminal fecal content in a linear structure (Fig. 1). Within a week of induction of colitis with a hapten, the biofilm was severely disrupted in both mice and rats (30). The microbiota was disorganized and heterogeneous, with different size clusters of bacteria. Fragments of these dysbiotic microbiota biofilms were also in close contact with the host tissue, and there was clear evidence of translocation of bacteria into the lamina propria, in a manner reminiscent of what was recently reported in the intestine of patients with IBD (37). Extensive depletion of epithelial mucus granules was also evident.

Experiments with mice deficient of one of the key enzymes for synthesis of H$_2$S, cystathionine $\gamma$-lyase (CSE), provided the initial indication that endogenous H$_2$S played a role in regulating epithelial mucus production (30). In contrast to what was observed in wild-type mice, the CSE-deficient mice exhibited mild colonic inflammation with a thinner-than-normal inner mucus layer. Despite evidence of a linear biofilm, there were bacterial aggregates in close contact with the epithelium. Very similar effects were seen in rats treated with an inhibitor of CSE activity ($\beta$-cyano-alanine; BCA): the biofilm that was fragmented, with bacteria in close contact with the colonic epithelium, and granulocytes were present within the biofilm and the lumen (Fig. 1). The depletion of epithelial mucus granules was much more evident in rats treated with the H$_2$S synthesis inhibitor than in vehicle-treated rats. Taken together, the experiments with CSE-deficient mice and with rats treated with a CSE inhibitor suggest that CSE-derived H$_2$S makes important contributions to the promotion of colonic microbiota biofilm
formation and stability, and contributes to enhanced mucus barrier function and epithelial integrity (30).

Additional evidence to support this hypothesis comes from studies of the effects of an \textsuperscript{1}H\textsubscript{2}S donor in experimental colitis. In rats with colitis, intracolonic administration of DADS (twice daily) for one week resulted in a significant acceleration of the resolution of colitis, which included acceleration of the restoration of linear biofilm organization and reduced bacterial translocation (30). After DADS treatment, a clear mucus layer separated the epithelium from the microbiota biofilm (as observed in healthy controls) (Fig. 2). When treatment with DADS was extended from 7 to 14 days, the mucus granule number per intestinal crypt increased significantly, to a level greater than that in healthy controls.

To further investigate the concept that \textsuperscript{1}H\textsubscript{2}S can enhance formation of biofilms, \textit{in vitro} studies were performed with human-derived intestinal biofilms (30). These biofilms were exposed to various concentrations of \textsuperscript{1}H\textsubscript{2}S donors (NaHS or DADS). At concentrations of 1 and 10 µM, exposure to either of the \textsuperscript{1}H\textsubscript{2}S donors resulted in a higher metabolic activity (a reflection of increased numbers of bacteria in the biofilm) and increased biomass (likely due to increased quantity of cells and proteins within the biofilm).

\textbf{Can \textsuperscript{1}H\textsubscript{2}S Modify the Microbiota?}

\textsuperscript{1}H\textsubscript{2}S donors derived from garlic have been shown to exert antimicrobial effects on planktonic Gram-positive and Gram-negative bacteria (12,26,34). We examined the effects of twice daily administration of an \textsuperscript{1}H\textsubscript{2}S donor (DADS) to rats at doses of 10 or 30 mmol/kg. These doses of DADS were ineffective and effective, respectively, in reducing the severity of NSAID-induced damage in the rat stomach (21). We observed that administration of the higher doses
of DADS resulted in significant shifts in the intestinal microbiota in rats (demonstrated via DNA extraction and denaturing gradient gel electrophoresis; DGGE) (Fig. 3). In the rats treated with the lower dose of DADS, the microbiota remained similar to that in vehicle-treated rats. None of the doses of DADS significantly changed the total numbers of aerobic or anaerobic bacteria in the jejunum (17).

Significant changes in the intestinal microbiota following administration of NSAIDs are well documented (45). Generally, NSAIDs given at doses that can cause significant enteropathy cause a shift in the microbiome towards more Gram-negative organisms (38), and this contributes significantly to intestinal damage through TNFα- and TLR-4-dependent pathways (47). In contrast, administration of an H₂S-releasing NSAID to rats resulted in significantly smaller shifts in the intestinal microbiome (4). It is not clear if this is the cause or an effect of the greatly reduced intestinal injury with H₂S-NSAID administration versus conventional NSAID administration, but the observations from studies of other H₂S donors suggest the former. One of the observed changes that may contribute significantly to the reduced intestinal damage observed with H₂S-NSAIDs is a marked decrease in the cytotoxicity of bile as compared to that from rats treated with a conventional NSAID (4). Secondary bile acids are much more cytotoxic than primary bile acids, and the conversion of primary to secondary bile acids is driven by bacterial enzymes (2). As shown in Figure 3, a similar reduction in the cytotoxicity of bile was observed when rats were treated with an H₂S donor at a dose that also caused a marked shift in intestinal microbiota (5).

**Summary and Future Directions**
There is substantial evidence that H$_2$S, produced by enteric bacteria, host cells or administered exogenously, can affect intestinal microbiota in a variety of ways. As well as reducing GI inflammation and injury, and promoting repair, exogenous and endogenous H$_2$S can positively affect many aspects of bacterial-epithelial interactions. There is evidence suggesting potential for the use of H$_2$S donors to favourably modulate the intestinal microbiota. Promotion of biofilm formation and integrity by H$_2$S donors is an important aspect, particularly in the context of improved treatments for disorders such as ulcerative colitis and Crohn’s disease.

It is also becoming increasingly clear that in addition to probiotics and antibiotics, several classes of drugs can dramatically and rapidly alter the GI microbiota, including some of the most widely used drugs (e.g., NSAIDs, proton pump inhibitors, histamine H2 receptor antagonists) (42). Studies in animals have provided proof-of-concept evidence that such detrimental changes can be reduced or prevented with H$_2$S donors. Moreover, the linking of H$_2$S-releasing groups to existing drugs is a promising approach to prevention of drug-induced dysbiosis and tissue injury, as has been demonstrated with the H$_2$S-releasing NSAID, ATB-346 (4,5,39).

**Abbreviations**

COX, cyclooxygenase; CSE, cystathionine gamma-lyase; BCA, β-cyanoalanine; DADS, diallyl disulfide; DGGE, denaturing gradient gel electrophoresis; FISH; fluorescent *in situ* hybridization; GI, gastrointestinal; H$_2$S, hydrogen sulfide; NSAID, nonsteroidal anti-inflammatory drug; PPI, proton pump inhibitor; TLR, toll-like receptor; TNF, tumour necrosis factor.
REFERENCES


FIGURE LEGENDS

Figure 1: Administration of an H₂S donor, diallyl disulfide (DADS), restored the biofilm in rats with hapten-induced colitis. After induction of colitis, groups of 4-5 rats each were treated daily with DADS (0.5 ml of 30 µM) or vehicle for 7 days. Fluorescent in situ hybridization was performed on colonic sections from the rats. The image shown is representative of what was observed in all rats receiving the treatments. Scale bars represent 50 µM. Dashed lines indicate the limit of the mucosa. The asterisk denotes translocated bacteria in the lamina propria. This figure was previously published (30) and is used in this paper with permission of the original publisher.

Figure 2: Administration of an inhibitor of endogenous H₂S synthesis in healthy mice and mice with hapten-induced colitis exacerbates inflammation and markedly alters the intestinal microbiota biofilm. Fluorescent in situ hybridization was performed on colonic sections from C57Bl/6 mice 7 days after induction of colitis with dinitrobenzene sulfonic acid. Representative images (from a total of 5 mice/group) are shown for (A) healthy controls, (B) colitis treated daily with vehicle, (C & D) colitis treated daily with β-cyanoalanine (BCA; 50 mg/kg). BCA is an inhibitor of H₂S synthesis. Host nuclei were coloured red while FISH-positive cells are green. Scale bars represent 25 µM (A & C) or 50 µM (B & D). Dashed lines indicate the limit of the mucosa. The asterisk denotes translocated bacteria in the lamina propria. This figure was previously published (30) and is used in this paper with permission of the original publisher.
Figure 3: **Panel A:** Naproxen treatment markedly increased the cytotoxic effects of bile on intestinal epithelial cells (IEC-6) (**p<0.001). Bile collected from rats that had been co-treated with naproxen and an H$_2$S donor (diallyl disulfide; DADS) exhibited reduced cytotoxicity, in a dose-dependent manner (\$p<0.05; \$\$\$ p<0.001). The rats were treated orally twice daily for 2 days with naproxen at 20 mg/kg, and with vehicle or DADS. **Panel B:** Denaturing gradient gel electrophoresis (DGGE) analysis of intestinal microbiota samples from rats treated with naproxen (20 mg·kg$^{-1}$) plus vehicle or DADS (30 mmol/kg). Treatment with DADS (blue) caused a mark shift in microbiota relative to vehicle-treated (black). **Panel C:** Using a resampling technique (majority unweighted-pair-group method with arithmetic mean algorithm), the dendrogram clustering observed in Panel B was confirmed, indicating a robust difference in microbiota composition between groups (vehicle-treated are black, DADs-treated are blue). Each group consisted of 5 rats. Data were analyzed with Dunnett’s multiple comparison test (cytotoxicity). This figure was previously published (5) and is used in this paper with permission of the original publisher.