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Hydrogen sulfide: an agent of stability at the microbiome-mucosa interface

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INTRODUCTION

Over the past 15 years, our understanding of the roles of the gaseous mediator hydrogen sulfide (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 defense, affecting such basic processes as mucosal blood flow, bicarbonate and mucus secretion, and endothelial-leukocyte interactions (13, 19, 49). Important roles for H$_2$S have also been demonstrated in visceral pain (7, 8, 10, 11) 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 such as ulcerative colitis, Crohn’s disease, and nonsteroidal anti-inflammatory drug (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 H₂S modulate the microbiota? Does H₂S affect the interactions between microbiota and the epithelium?

Several recent studies have provided evidence that exogenous and endogenous H₂S 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 coadministration of H₂S. Indeed, a novel class of NSAIDs has been developed to exploit these beneficial effects of H₂S in countering the damaging effects of conventional NSAIDs (7), which are among the most widely used drugs.

**IS LUMINAL H₂S TOXIC TO THE INTESTINAL EPITHELIUM?**

H₂S is produced by a wide range of enteric bacteria, primarily of the γ-Proteobacteria genera [readers are referred to a comprehensive review by Linden (25)]. H₂S concentrations in the cecum and rectum can reach 40 μM (36), and as much as 250 μM in the colon (1). There have been a wide range of studies using different types of transformed or nontransformed epithelial cells to determine their responsiveness to a range of concentrations of H₂S, including concentrations well above 250 μM (25). Not surprisingly, there are discrepant findings from these studies, with no clear and consistent evidence for toxic effects of H₂S on epithelial cell integrity and considerable variability in terms of the effects of H₂S on epithelial cell proliferation (25). Linden (25) noted that H₂S (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 antiproliferative and chemopreventive effects of H₂S in vitro and in vivo (9, 24, 32, 33, 46).

There has been speculation of potential links between bacteria-derived H₂S 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 H₂S donors were administered to rodents or dogs for periods of up to 2 wk but have not observed any evidence of tissue injury. Even with twice-daily administration of a garlic-derived H₂S 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 was there any mucosal inflammation (5). On the contrary, with administration of H₂S 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, ~50% was rapidly converted to H₂S.

On the other hand, a recent study of pediatric Crohn’s disease provided compelling evidence for a role of H₂S in promoting damage in a subset of patients who had a genetic defect in mitochondrial oxidation of H₂S (31). Interestingly, those patients also had substantially increased numbers of H₂S-producing bacteria in their intestinal lumen (31). We observed a similar increase in H₂S-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 H₂S is an important metabolic fuel for the epithelial cells that line the GI tract (17, 29). Colonic epithelial cells are particularly efficient in oxidizing H₂S, 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 H₂S (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 H₂S from luminal bacteria to gain access to the lamina propria.

**PROTECTIVE AND REPARATIVE EFFECTS OF H₂S**

H₂S 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, H₂S donors have been shown to promote resolution of colitis in several animal models (14, 18, 23, 43). Endogenous H₂S production is markedly elevated at sites of mucosal injury, contributing significantly to promotion of healing (40, 43). Moreover, rates of oxidation of H₂S are specifically and substantially reduced at sites of epithelial/mucosal injury (14). On the other hand, inhibition of H₂S synthesis leads to increased susceptibility to mucosal injury, impairment of healing, increased proinflammatory cytokine expression, reduced expression of cyclooxygenase-2 (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, AND 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 endog-
ogenous H\(_2\)S synthesis vs. administering exogenous H\(_2\)S (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 [\(\beta\)-cyanoalanine (BCA)]: the biofilm 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 H\(_2\)S donor in experimental colitis. In rats with colitis, intracolonic administration of DADS (twice daily) for 1 wk 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 H\(_2\)S can enhance formation of biofilms, in vitro studies were performed with human-derived intestinal biofilms (30). These biofilms were...
exposed to various concentrations of H₂S donors (NaHS or DADS). At concentrations of 1 and 10 μM, exposure to either of the H₂S 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).

**CAN H₂S MODIFY THE MICROBIOTA?**

H₂S 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 H₂S 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; 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 toward more gram-negative organisms (38), and this contributes significantly to intestinal damage through TNF-α- and toll-like receptor 4 (TLR4)-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 whether this is the cause or an effect of the greatly reduced intestinal injury with H₂S-NSAID administration vs. 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 compared with 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 Fig. 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 H₂S, produced by enteric bacteria or 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 H₂S can positively affect many aspects of bacterial-epithelial interactions. There is evidence suggesting potential for the use of H₂S donors to favorably modulate the intestinal microbiota. Promotion of biofilm formation and integrity by H₂S 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, and histamine H₂ receptor antagonists; 42). Studies in animals have provided proof-of-concept evidence that such detrimental changes can be reduced or prevented with H₂S.

Fig. 3. 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 cotreated with naproxen and an H₂S 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. B: denaturing gradient gel electrophoresis analysis of intestinal microbiota samples from rats treated with naproxen (20 mg/kg) plus vehicle or DADS (30 mmol/kg). Treatment with DADS (blue) caused a marked shift in microbiota relative to vehicle-treated rats (black). C: using a resampling technique (majority unweighted-pair-group method with arithmetic mean algorithm), the dendrogram clustering observed in B was confirmed, indicating a robust difference in microbiota composition between groups (black, vehicle-treated rats; blue, DADS-treated rats). Each group consisted of five rats. Data were analyzed with Dunnett’s multiple-comparison test (cytotoxicity). [From Blackler et al. (5).]
REFERENCES


29. Minoun S, Andriamihaja M, Chaumontet C, Atanasu C, Benamouzig R, Blouin JM, Tomé D, Bouillaud F, Blachier F. Detoxification of donors. Moreover, the linking of H2S-releasing groups to existing drugs is a promising approach to prevention of drug-induced dysbiosis and tissue injury, as has been demonstrated with the H2S-releasing NSAID, ATB-346 [2-(6-methoxynaphthalen-2-yl)-propionic acid 4-thiocarbamoyl phenyl ester; 4, 5, 39].


