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INVITED REVIEW

Gaseous Mediators in Gastrointestinal Mucosal Defense and Injury

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Abstract Of the numerous gaseous substances that can act as signaling molecules, the best characterized are nitric oxide, carbon monoxide and hydrogen sulfide. Contributions of each of these low molecular weight substances, alone or in combination, to maintenance of gastrointestinal mucosal integrity have been established. There is considerable overlap in the actions of these gases in modulating mucosal defense and responses to injury, and in some instances they act in a cooperative manner. Each also play important roles in regulating inflammatory and repair processes throughout the gastrointestinal tract. In recent years, significant progress has been made in the development of novel anti-inflammatory and cytoprotective drugs that exploit the beneficial activities of one or more of these gaseous mediators.

Keywords Nitric oxide · Hydrogen sulfide · Carbon monoxide · Ulcer · Colitis · Inflammation · Inflammatory bowel disease · Mucosal defense

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Introduction

The gastrointestinal (GI) tract shows a remarkable resilience to damage induced by the beverages and foods that we ingest, which can have a wide range of osmolarity, pH and temperature. It is rare for the GI mucosa to succumb, in a clinically significant way, to the detergent actions of bile, to the proteolytic actions of gastric, intestinal or pancreatic secretions, to many of the different types of drugs that we ingest, or to the potentially harmful actions of certain enteric microbes colonizing the GI tract [1]. This resistance to tissue damage is collectively referred to as "mucosal defense" and it consists of a range of different factors that act to prevent injury and to promote rapid repair when injury occurs. In recent years, we have come to a better understanding of the mechanisms underlying GI mucosal defense, as well as the systems for rapid and orderly repair of damage. Intertwined with these is the crucial role of inflammation as a predominantly beneficial process that can become detrimental when dysregulated. Such dysregulation can be triggered by certain drugs or infections. Many different chemical mediators participate in mucosal defense, inflammation and repair. In this paper, we describe some of the key components of GI mucosal defense and the evidence for significant roles of three gaseous mediators in GI mucosal defense (nitric oxide (NO), hydrogen sulfide (H_2S) and carbon monoxide (CO)) in modulating the various elements of mucosal defense. We also discuss the potential for drug development based on these gaseous mediators that is specifically related to enhancement of GI mucosal defense, anti-inflammatory activity and/or promotion of healing.

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Gaseous Mediators of GI Mucosal Defense

NO is the most studied of the three gaseous mediators. Numerous drugs have been developed for which the release of NO accounts for a significant part of their actions, including nitroglycerin. The 1998 Nobel Prize for Physiology/Medicine was awarded for the discovery that NO accounted for the vasodilatory actions that had previously been attributed to 'endothelium-derived relaxing factor' [2]. NO is produced by many different types of cells in the GI tract and performs a wide range of functions that include many pertinent to GI mucosal defense (see below). There are three enzymes primarily responsible for NO production: NOS-1 ("neuronal"), NOS-2 ("inducible") and NOS-3 ("endothelial") (Fig. 1). NOS-1 and NOS-3 are
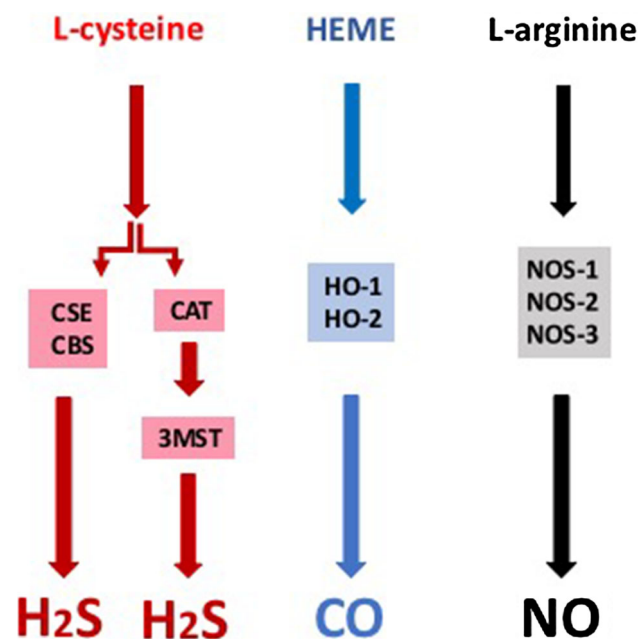

Fig. 1 Pathways of synthesis of three gasotransmitters (hydrogen sulfide, carbon monoxide and nitric oxide; H_2S, CO and NO, respectively). H_2S can be produced from L-cysteine through three separate pathways. Cystathionine gamma-lyase (CSE) and cystathionine ß-synthase (CBS) are widely expressed and considered the major enzymatic courses of H_2S, producing pyruvate and L-serine as byproducts, respectively. The sequential actions of cysteine aminotransferase (producing 3-mercaptopyruvate from L-cysteine) and 3-mercaptopyruvate sulfurtransferase (3MST) can also produce H_2S. A fourth pathway of H_2S synthesis, not shown, is that from D-cysteine via the enzymes 3MST and D-amino acid oxidase [88]. CO is produced from heme via the actions of hemoxygenase-1 (HO-1; constitutively expressed) or hemoxygenase-2 (HO-2; inducible expression), with biliverdin and iron produced as by-products of this reaction. NO can be produced from L-arginine and NADPH by three forms of NO synthase, yielding L-citrulline as a by-product. NOS1 and NOS 3 are constitutively expressed, while NOS2 is inducible. The three NOS isoforms were previously known as neuronal NOS (NOS1), inducible NOS (NOS2) and endothelial NOS (NOS3)

generally regarded as being constitutively expressed, though their expression can be modulated [2].

H_2S has long been known as a potentially toxic gas with a pungent smell, but studies by Kimura and Abe in 1996 [3, 4] and Wang et al. in 2001 [5] drew attention to physiological roles of this molecule in the nervous and circulatory systems, respectively. These studies stimulated a burst of research into the role of this gaseous mediator in many cells, tissues and organs, and suggested several potential clinical applications for drugs that deliver H_2S or inhibit synthesis of H_2S [6]. There are several enzymatic pathways for H_2S synthesis in eukaryotes (Fig. 1).

H_2S is implicated in the origin of life, and served as a major energy source for organisms for most of the time since then (oxygen replaced H_2S as the major energy source only in the last ~ 600 million years) [7]. Interestingly, mitochondria can utilize H_2S to drive adenosine triphosphate (ATP) production. In mammals, the cells that are most efficient at H_2S-driven energy production are the epithelial cells lining the GI tract [8, 9].

Like H_2S, CO is known primarily as a pollutant, but in the past two decades its role as a gaseous mediator in many biological processes have become clear [10]. Production of CO from heme occurs via the enzyme hemoxygenase-1 (HO-1; constitutive) and hemoxygenase-2 (HO-2; inducible) (Fig. 1). Most current literature with respect to the GI tract is focused on CO derived from HO-1, which is constitutively expressed in many types of cells in the GI tract, including epithelial cells, mononuclear cells, endothelial cells, and smooth muscle [11]. Biliverdin is the other product of this reaction. The metabolism of heme, through the actions of HO-1, has beneficial effects (e.g., reduction of oxidative stress) since heme can be cytotoxic. HO-2 in the GI tract is primarily involved in modulating smooth muscle membrane potential and neuromodulation [12] CO also acts as a signaling molecule, influencing a wide range of biological processes and exerting cytoprotective and anti-inflammatory effects [11]. CO is much more biologically stable than NO and H_2S since it is not a free radical. Thus, unlike the other two gaseous mediators, it is able to produce effects distant to where it is produced [13].

A significant limitation for exploring the effects of CO in vivo has been the lack of a range of CO donors that are proven to be useful for experimental studies. The most useful tools available thus far have been a group of molecules called "CORMs" (CO-releasing molecules), developed by Motterlini and colleagues [10]. Some beneficial effects of CORMs appear to be due to down-regulation of expression of NOS2 [14], an example of cross-talk among the gaseous mediators.

Mucus and Bicarbonate Secretion

Mucus is secreted by GI epithelial cells and goblet cells, and acts as a lubricant to reduce physical abrasion of the mucosa. It also plays important roles in protection of the mucosa from damage induced by acid and other luminal toxins. It provides an important physical barrier to bacteria, reducing bacterial adherence to the epithelium and thereby reducing translocation into the lamina propria.

An increase in mucus thickness is a normal defensive response to luminal insults. As well as providing a barrier to translocation of microbes into the sub-epithelial space, mucus traps secreted bicarbonate and plasma on the surface of the epithelium, which provides a near-neutral pH microenvironment that is conducive to epithelial protection and repair. All three gaseous mediators can influence mucus secretion in the GI tract. NO stimulates epithelial mucus secretion via activation of guanylyl cyclase in epithelial cells [15], and carbachol-induced gastric mucus release is also mediated via NO [16]. H_2S and CO can also increase gastric mucus secretion [17–19]. The effects of H_2S on colonic mucus secretion are particularly interesting and complex. In healthy rats, microbiota form linear biofilms in the colon. During colitis, the biofilms become fragmented, and production of mucus granules is markedly reduced [19]. Endogenous H_2S production stimulates mucus production and promotes establishment of microbiota biofilms. Moreover, administration of an H_2S donor to rats with colitis restores the microbiota biofilm as well as increasing the production of mucus by the epithelium [19].

The GI epithelium also defends itself against bacterial invasion via the secretion of fluid into the lumen. This reduces bacterial adherence, and the secretion of fluid also dilutes any noxious substances in the lumen. Fluid secretion across the GI epithelium is mainly osmotically driven by the active transport of chloride ions into the lumen. This process is regulated by numerous soluble mediators and neurotransmitters, including NO [20]. NO donors can stimulate chloride transport in the small intestine [21, 22]. However, the effects of NO on intestinal secretion are not always stimulatory: low concentrations of NO are stimulatory while high concentrations are inhibitory. NO also plays a key role in some of the long-term impairment of epithelial secretion that can be observed after a bout of intestinal inflammation. [23, 24].

Secretion of bicarbonate by gastric and duodenal epithelial cells is a very important component of mucosal defense, and it can be significantly modulated by the three gaseous mediators [25]. In the resting state, there is a basal level of gastroduodenal bicarbonate secretion, but a significant increase is triggered when acid back-diffuses into the mucosa. All three of the gaseous mediators can stimulate bicarbonate secretion [26, 27]. Thus, duodenal bicarbonate secretion is increased after administration of an H_2S donor, and diminished by an inhibitor of endogenous H_2S synthesis. Suppression of H_2S synthesis also leads to an enhancement of acid-induced duodenal damage [25]. Administration of an H_2S-releasing derivative of naproxen (ATB-346) resulted in a marked ($\sim 50\%$) decrease in gastric acidity, with the mean pH of gastric juice increasing from 1.5 to 2.1, and a $>80\%$ decrease in the volume of secretion [28]. Similar results were reported from studies using another H_2S donor [29]. The decrease in gastric acidity observed after administration of an H_2S donor did not appear to be due to suppression of acid secretion [17, 28]. Regulation of bicarbonate and acid secretion by H_2S may contribute significantly to the gastroprotective effects of H_2S-releasing NSAIDs [28].

With respect to CO, administration of CORM-2 dose-dependently stimulated duodenal bicarbonate secretion [26]. Suppression of NO synthesis did not affect this response, but suppression of cyclooxygenase (COX) activity significantly attenuated CO-stimulated bicarbonate secretion, suggesting a role for prostanoids in mediating the effects of CO. Exposure of the duodenal mucosa to acid resulted in a significant increase in expression of HO-1, but not HO-2 [26].

Acid-induced duodenal bicarbonate secretion is also mediated in part via local release of NO and via generation of prostaglandins [27]. Indeed, both guanylin, an endogenous activator of guanylate cyclase, and NOR-3, a NO donor, increased bicarbonate secretion [27].

Acid Secretion

Gastric acid is a defensive factor in that it is bactericidal. The importance of acid in this regard is evident from studies demonstrating that marked suppression of acid secretion results in increased bacterial colonization of the stomach [30]. Moreover, suppression of gastric acid secretion can lead to dramatic, biologically significant alterations in the small intestinal microbiota, increasing the risk of enteric infection [31] and contributing to several disorders, including microscopic colitis, inflammatory bowel disease and small intestinal bacterial overgrowth [32–34]. For example, administration of proton pump inhibitors or histamine H2 receptor antagonists, prescribed with a primary goal of reducing NSAID-induced upper GI damage, markedly increase the severity of small intestinal damage [35–38]. Studies in laboratory animals demonstrated that treatment with a proton pump inhibitor led to a marked depletion of Actinobacteria and *Bifidobacteria* species in the small

intestine [35], and this was accompanied by significant increases in the cytotoxicity of bile [39, 40]. Replenishment of intestinal *Bifidobacteria* levels restored to normal the intestinal resistance to NSAID-induced injury [35].

The three gaseous mediators have minimal effects on gastric acid secretion [17, 41, 42].

Mucosal Blood Flow and Microcirculation

Regulation of blood flow is critical to mucosal defense. This is particularly so in the stomach and duodenum, where back-diffusion of acid can cause extensive damage and bleeding. However, a well-characterized and rapid reactive hyperemic response to acid back-diffusion can prevent or limit mucosal injury. This response is initiated by sensory afferent nerves underlying the epithelium, which respond immediately to entry of acid into the lamina propria [43]. Release of calcitonin gene-related peptide (CGRP) from these neurons results in immediate dilation of submucosal arterioles facilitating the dilution and buffering of the back-diffused acid [44]. The CGRP vascular response is mediated by NO; administration of an inhibitor of NO synthesis abolishes the reactive hyperemic response, resulting in a marked increase in the susceptibility of the mucosa to damage [45]. There is strong evidence for CO and H_2S contributing significantly to maintenance of tissue perfusion in the upper GI tract, particularly when acid back-diffusion has been triggered by agents such as non-steroidal anti-inflammatory drugs (NSAIDs) or ethanol [46–48].

The GI tract, particularly distal to the duodenum, is essentially in a state of chronic, low-grade inflammation. This is due to the ongoing interaction (and trans-epithelial migration) of luminal bacteria and their products with the mucosal immune system. Leukocytes can be stimulated to extravasate from mucosal blood vessels by chemotaxins that are released from bacteria, and this process can result in damage to the blood vessels and surrounding tissue, and further generation of chemotaxins. NO, H_2S and CO all play roles in modulating leukocyte adherence to the vascular endothelium, and as discussed above, maintenance of blood flow to the tissue. For example, inhibition of NO synthesis results in a marked increase in leukocyte adherence to the endothelium [49]. NO has been shown to inhibit expression of the ß-2 adhesion molecules on neutrophils [50] and P-selectin on the vascular endothelium [51]. Adherence of leukocytes to the vascular endothelium in response to stimulation with a chemotactic factor can be suppressed by administration of an NO donor [52]. Inhibition of H_2S synthesis can trigger leukocyte adherence to the vascular endothelium, while H_2S donors have inhibitory effects on this process [53], as well as suppressing leukocyte infiltration in models of inflammation [54]. CO can also inhibit leukocyte adherence [55]. It has been suggested that the reduction of mucosal injury elicited by inducers of HO-1 occur as a consequence of inhibition of leukocyte infiltration [56].

Cytoprotection

NO donors exert protective effects in the GI tract through a range of mechanisms that include increasing mucosal blood flow, inhibiting leukocyte adherence to the vascular endothelium, and stimulating mucus and bicarbonate secretion [57]. Some of the beneficial effects of NO may be attributable to CO, since NO can induce heme oxygenase [58]. NO-releasing NSAIDs demonstrated greatly reduced damaging effects in the GI tract in extensive animal testing and in early clinical trials [28]. However, in a pivotal trial of an NO-releasing derivative of naproxen, a statistically significant reduction of upper GI damage was not achieved [59].

Like NO, H_2S has been shown to be cytoprotective for the GI tract in animal studies [60]. For H_2S, there is considerable overlap with NO in terms of the protection afforded by H_2S donors and the exacerbation of damage with drugs that suppress H_2S synthesis [47, 48]. An H_2S-releasing NSAID has been shown to be very effective and potent in reducing osteoarthritis-related pain [61] and recently a phase 2 clinical trial to examine its safety in the GI tract was initiated.

HO-1 and CO have been shown to exert beneficial effects in animal models of gastric ulceration, including NSAID-induced mucosal injury [16, 48, 62, 63] and the enteropathy associated with post-operative ileus [64]. CO has also been observed to protect the stomach from ethanol-induced lesions, mediated in part by NO and by COX-1-derived prostaglandin synthesis [49].

Healing and Resolution of Inflammation

Given the many potentially damaging substances present in the lumen of the GI tract, mechanisms to resist damage are backed up by mechanisms for rapid repair when damage occurs. The gaseous mediators participate significantly in these repair processes. Inhibitors of NO or H_2S synthesis significantly retard gastric ulcer healing and healing of damaged tissue in models of colitis [65–69]. On the other hand, NO, CO and H_2S donors accelerate the healing of these lesions [66–68, 70]. The pro-healing effects of these gases are mediated in part by increased epithelial cell migration and proliferation, and through enhancement of collagen deposition by fibroblasts [67, 68, 70, 71]. Another mechanism through which these vasodilatory gases can

accelerate ulcer healing is via maintenance of blood flow at the margin of the wound. Ulcer healing requires proliferation and differentiation of epithelial cells at the ulcer margin, and that in turn is dependent upon adequate blood flow—reductions of blood flow in these circumstances result in a retardation of ulcer healing [72]. Ulcer healing also requires the growth of new blood vessels in the ulcer margin, through the process of angiogenesis, and all three of the gaseous mediators are stimulants of this process [73].

The anti-inflammatory and pro-resolution effects of the gaseous mediators are also evident in the more distal GI tract, particularly in colitis. H_2S has been shown to play an important role in regulating prostaglandin synthesis and expression of COX-2 in the GI tract [68] (Fig. 2). This has a significant impact on maintenance of mucosal integrity and the capacity to rapidly respond to injury. Thus, H_2S promotes the expression of COX-2 in the GI tract, while inhibitors of H_2S synthesis reduce this "constitutive" expression of COX-2 [68]. However, when there is chronic inflammation, such as in colitis, H_2S can dampen COX-2 expression, leading to reduced levels of several pro-inflammatory cytokines and chemokines [52, 68, 69, 74]. Thus, H_2S plays important roles in promoting resolution of inflammation in the GI tract [19, 52, 69, 74]. H_2S synthesis is specifically up-regulated at sites of ulceration in the colon, and degradation of H_2S is down-regulated; thus, levels of H_2S increased in ulcerated tissue, where rapid healing is most needed [69]. Inhibition of H_2S synthesis in models of colitis results in a marked exacerbation of damage and delay of healing [68, 74].

H_2S is unique among the three major gaseous mediators in that it can substitute for oxygen in driving the generation of ATP in mitochondria. Thus, H_2S acts as a 'rescue molecule' in many circumstances, helping to preserve tissue integrity and function while repair occurs. The epithelial cells of the GI tract have been reported to be the most efficient at using H_2S to drive mitochondrial ATP production [8, 9, 75]. The efficient oxidation of bacteria-derived H_2S by the intestinal epithelium is also a means of limiting H_2S from entry to the mucosa [8, 9, 75];
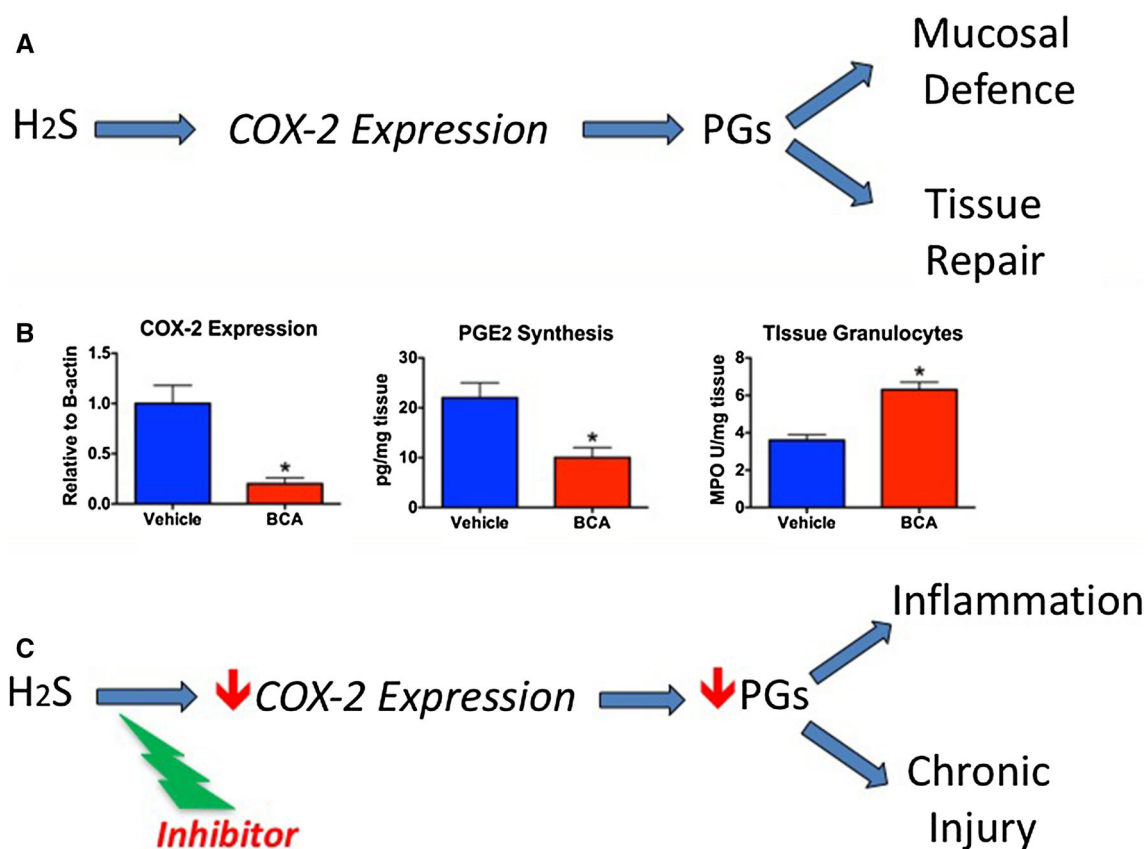

Fig. 2 Interactions of hydrogen sulfide (H_2S) and cyclooxygenase-2 (COX-2) regulate gastrointestinal (GI) mucosal defense, inflammation and repair. In healthy rats (panel A) there is low-grade inflammation in the GI tract, and constitutive H_2S synthesis stimulates 'basal' COX-2 expression, and synthesis of prostaglandins (PG) that contribute to mucosal defense and repair. Panels B and C: pharmacological inhibition of H_2S synthesis with ß-cyanoalanine (BCA) results in a significant reduction of COX-2 expression and prostaglandin E_2 synthesis in the GI tract, as well as significant increases in tissue granulocyte numbers ($p < 0.05$ versus vehicle-treated). Thus, suppression of H_2S synthesis removes a basal cytoprotective factor, which results in impaired mucosal defense and chronic injury and inflammation [89]

nevertheless, approximately half of the H_2S that can be measured in the body is derived from bacteria [76, 77].

The role of NO in treatment and pathogenesis of IBD is very complicated, with some studies showing beneficial effects of treatment with NO donors [52, 78] and others showing beneficial effects of inhibition of endogenous NO synthesis [79, 80]. Expression and activity of NOS2 are markedly increased in colitis and appear to drive inflammation and, in particular, expression of pro-inflammatory cytokines [81]. Selective suppression of NOS2 activity has been shown to promote resolution of intestinal inflammation [79, 80]. However, other studies suggest that NO derived from NOS2 drives resolution of colitis [82] and is also responsible for long-lasting impairment of intestinal secretory function, which may underlie post-inflammatory gut dysfunction [23, 24]. It is likely that much of this discrepancy of results and conclusions in the literature is attributable to 'off-target' effects of the pharmacological tools that have been used, and variable responses of the many different animal models that have been employed.

Administration of CORM-2 [83] or CO given via inhalation [84] have also been shown to produce beneficial effects in experimental colitis, including marked reductions in pro-inflammatory cytokine expression, granulocyte infiltration, crypt damage and ulcers [83, 84]. The reduction of pro-inflammatory cytokine expression may be attributable to CO-induced inhibition of NF-κB activity [84, 85]. CO derived from HO-1 may also ameliorate intestinal inflammation by promoting the clearance of bacteria at sites of injury [86]. Innate immune cells exposed to CO have been shown to have enhanced bactericidal activity [86]. Interestingly, there are enteric bacterial hemoxygenase homologs that may serve as a significant source of CO in the GI tract, possibly playing a role in promotion of homeostasis [86].

Future Directions

There is an extensive body of work demonstrating important roles of NO, H_2S and CO in modulating a wide range of physiological processes, and suggesting their utility as therapeutics. With respect to the GI tract, there are many potential clinical applications for gaseous mediators if they can be delivered to the target tissues in appropriate amounts. Most of the evidence that has been generated thus far suggest important roles for these gaseous mediators in promoting GI tissue integrity, modulation of inflammation and acceleration of tissue repair. Evidence that H_2S donors can modify the intestinal microbiome, in a positive manner, is particularly intriguing [19, 39, 40]. A limitation of most studies of gaseous mediators in the GI tract is that very simple donor molecules have been used that release the

mediator but in an uncontrolled and non-targeted manner. The future of this field with respect to drug development will require patented molecules that will release the gaseous mediator at a rate appropriate for promoting health, and at the specific locations where the effects of the mediator are most needed. This will be a significant challenge, but there have been some successful developments in recent years, one example being H_2S donors that specifically target mitochondria [87].

Compliance with ethical standards

Conflict of interest Dr. Wallace is the founder and Chief Scientific Officer of Antibe Therapeutics Inc., which is developing hydrogen sulfide-releasing drugs.

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