Background & Aims: Hydrogen sulfide (H₂S) is an endogenous gaseous mediator of mucosal defense with antiinflammatory effects that promote ulcer healing. The effects of H₂S during the pathogenesis of colitis have not been established. We analyzed the contribution of H₂S to inflammation and ulceration of the colon in a rat model of colitis. Methods: Colitis was induced by intracolonic administration of trinitrobenzene sulfonic acid. The ability of the colon to synthesize H₂S was studied over the course of the resolution of the colitis. Expression of 2 enzymes involved in the synthesis of H₂S and the effects of inhibitors of these enzymes were examined. We also examined the effects of H₂S donors on the resolution of colitis. Results: The capacity for the colon to produce H₂S increased markedly over the first days after induction of colitis and then declined toward control levels as the colitis was resolved. Inhibition of colonic H₂S synthesis markedly exacerbated the colitis, resulting in significant mortality. Inhibition of H₂S synthesis in healthy rats resulted in inflammation and mucosal injury in the small intestine and colon along with down-regulation of cyclooxygenase-2 messenger RNA expression and prostaglandin synthesis. Intracolonic administration of H₂S donors significantly reduced the severity of colitis and reduced colonic expression of messenger RNA for the proinflammatory cytokine tumor necrosis factor α. Conclusions: In rats, H₂S modulates physiological inflammation and contributes to the resolution of colitis.

Background & Aims: Hydrogen sulfide (H₂S), although often regarded mainly as a toxin and industrial pollutant, is produced throughout the body, with normal plasma concentrations being in the 30 to 100 μmol/L range. H₂S has recently been identified as an endogenous vasodilator, neuromodulator, and antiinflammatory mediator. It contributes significantly to gastric mucosal defense, promotes the healing of gastric ulcers in rodents, and has been implicated as a regulator of intestinal secretion in guinea pigs. Two of the key enzymes for synthesis of H₂S have been identified in the colon of guinea pigs and humans (expressed in >90% of submucous and myenteric neurons) and in the stomach and small intestine of rodents. These findings all suggest important physiologic and pathophysiologic roles of H₂S in the gastrointestinal tract.

Concentrations of H₂S in the lumen of the colon have been reported to be extremely high relative to physiologic concentrations in the body. However, there is disagreement as to whether or not the concentrations of free H₂S really reach the millimolar range that has been reported. A specific role of H₂S in the context of inflammatory bowel disease (IBD) has been suggested, but there are diametrically opposed views of this role. On the one hand, there have been suggestions for many years that high levels of H₂S produced in the lumen of the colon by bacteria could contribute to colitis and possibly to colon cancer. It has been suggested that luminal H₂S may impair oxidation of n-butyrate by colonocytes and that this could lead to impaired barrier function and thereby contribute to the pathogenesis of ulcerative colitis. However, this specific effect of H₂S has been challenged, as has the potential contribution of H₂S to the pathogenesis of ulcerative colitis. Indeed, there is a lack of compelling evidence that H₂S causes damage to colonic epithelial cells; rather, colonocytes appear to be particularly well adapted to utilize H₂S as a metabolic fuel. Moreover, a beneficial effect of H₂S in the context of colitis was suggested by the recent demonstration of markedly improved antiinflammatory activity of a H₂S-releasing derivative of mesalamine in experimental colitis. Unlike mesalamine, the H₂S-releasing mesalamine derivative significantly reduced colonic expression of several proinflammatory cytokines (eg, tumor necrosis factor [TNF]-α, interferon [IFN]-γ), possibly a consequence of the reported ability of H₂S to inhibit activation of nuclear transcription factor (NF)-κB. In rats, H₂S donors...
were recently shown to significantly inhibit leukocyte adherence to the vascular endothelium, extravasation of leukocytes in response to zymosan, and paw edema induced by carrageenan. Moreover, endogenous H_{2}S appears to play a significant role in modulating inflammatory reactions, as indicated by the exacerbation of acute inflammatory reactions when inhibitors of H_{2}S were administered to rats.

The present study was performed to delineate better the possible role of H_{2}S in experimental colitis. The trinitrobenzene sulfonic acid (TNBS) model of colitis in rats is very well suited for this study because one can examine tissues immediately before and at defined times after induction of colitis and because the course of resolution of colitis in this model has been well characterized.

Colonic H_{2}S synthesis was measured over the course of a bout of colitis, as was expression of 2 key enzymes for H_{2}S synthesis. We then examined the effects of inhibitors of H_{2}S synthesis and of H_{2}S donors on the severity of colitis. Our findings point to an important contribution of endogenous H_{2}S to the resolution of colitis and suggest that this process can be accelerated via administration of exogenous H_{2}S. Moreover, administration to healthy rats of an inhibitor of endogenous H_{2}S synthesis led to significant colonic inflammation.

Materials and Methods

Animals

Male Wistar rats (200–225 g) were fed standard laboratory chow and water ad libitum. They were housed in plastic cages in a room with controlled temperature (22°C ± 1°C), humidity (65%–70%), and light cycle (12 hours light-dark). All experiments were conducted in accordance with the guidelines established by the Canadian Council of Animal Care, and the protocols have been approved by the Animal Care Committee at the University of Calgary. Colitis was induced via intracolonial administration of TNBS (45 mg/mL in 0.5 mL of 40% ethanol).

H_{2}S Synthesis

The principal pathways for H_{2}S synthesis in mammals involve conversion of L-cysteine to pyruvate, ammonium, and H_{2}S via either of 2 pyrophospho-dependent enzymes: cystathionine-γ-lyase (CSE) and cystationine-β-synthase (CBS). Groups of rats (n ≥ 6) were killed 6 hours to 28 days after TNBS administration for determination of H_{2}S synthesis and expression of CSE and CBS. The severity of colitis was scored using a system described in detail previously, which takes into consideration the presence and extent of inflammation and ulceration. Samples of the colon (full thickness) were taken from a region 3–4 cm proximal to the rectum. The samples were snap frozen in liquid nitrogen and stored at −80°C until the H_{2}S assay was performed. The method for measuring colonic synthesis of H_{2}S is modified slightly from that described by Qu et al. The standard curve for this spectrophotometric assay was constructed using various concentrations of NaHS.

CBS and CSE Expression

Western blot analysis was used to determine colonic expression of CBS and CSE in samples of colon from rats with colitis and healthy controls. The expression of CSE and CBS in a sample was normalized to the expression of β-actin. Immunohistochemical analysis of CSE and CBS expression was performed by routine techniques using paraffin-embedded samples of colon. Mouse monoclonal anti-CSE and anti-CBS antibodies (ABNOVA, Taipei, Taiwan) were used.

Effects of Inhibitors of Endogenous H_{2}S Synthesis

Effects of inhibitors of CBS and CSE were tested in vitro in the H_{2}S synthesis assay, using colonic tissue harvested from healthy controls and from rats with colitis (72 hours post-TNBS administration). We examined the effects of 2 inhibitors of CSE (β-cyanoalanine [BCA], 3 mmol/L; and propargylglycine [PAG], 3 mmol/L) and 1 inhibitor of CBS (O-carboxymethyl-hydroxylamine hemihydrochloride [CHH], 3 mmol/L). These concentrations of the drugs were selected based on pilot studies performed in our laboratory using rat colon. PAG and BCA differ in their mechanisms of inhibition of CSE, the former being an irreversible inhibitor and the latter a reversible inhibitor. Additional in vitro studies were performed in which the effects on H_{2}S synthesis of a combination of PAG and CHH were evaluated in samples taken at various times after induction of colitis (6 and 24 hours, and 3 and 7 days after TNBS administration).

We then performed in vivo studies in which groups of rats (n = 12) that had been given TNBS intracolonically to induce colitis or healthy controls were treated twice daily, intraperitoneally (IP), with 1 of the H_{2}S synthesis inhibitors for 7 days (BCA and PAG at 50 mg/kg, CHH at 20 mg/kg). Rats that survived until either day 7 or day 14 post-TNBS administration were killed, and the severity of colitis was blindly evaluated. Samples of colonic tissue were processed by routine techniques for histologic examination and for determination of myeloperoxidase (MPO) activity as a marker of granulocyte infiltration into the tissue.

Role of Adenosine Triphosphate-Sensitive K+ Channels

Some actions of H_{2}S are mediated, at least to some extent, via activation of adenosine triphosphate (ATP)-sensitive K+ (K_{ATP}) channels. Experiments were performed as described above, but the treatments included either an agonist of K_{ATP} (pinacidil, 10 mg/kg, IP) or an antagonist of these channels (glibenclamide, 10 mg/kg, IP).
**Effects of H₂S Donors**

Starting 1 hour after TNBS administration, groups of rats (n ≥ 6) were treated twice daily, intracolonically, with an H₂S donor (NaHS, 30 μmol/kg; or Lawesson’s reagent, 30 μmol/kg) or vehicle for a total of 4 days. The selection of doses of the H₂S donors was based on our prior experience with these drugs.³⁷ The rats were killed 2 hours after the final dose, and the severity of colitis was blindly evaluated.¹⁹ The thickness of the colon was measured with digital calipers. Samples of colonic tissue were processed for histology and for determination of expression of messenger RNA (mRNA) for TNF-α, COX-1, and COX-2 mRNA (see below). Additional samples were processed, as described previously, for determination of colonic prostaglandin E₂ synthesis³² and TNF-α protein levels³³ by enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical Ltd., Ann Arbor, MI, and R&D Systems, Minneapolis, MN, respectively).

**Real-time Reverse Transcription Polymerase Chain Reaction**

Samples of distal colonic tissue were excised, snap frozen in liquid nitrogen, and stored at −80°C until required for processing. RNA extraction was performed using the RNeasy Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. For gene expression studies, 2-step real-time reverse transcription polymerase chain reaction was utilized, as described previously.³⁴

**Statistical Analysis**

All data are expressed as the mean ± SEM. Groups of data were compared using a 1-way analysis of variance followed by the Dunnett multiple comparison test, or by the Mann-Whitney U test, where appropriate. A Gehan-Breslow-Wilcoxon test was used for comparisons of survival data. An associated probability (P value) of less than 5% was considered significant.

**Results**

**Colonic H₂S Synthetic Capacity Is Markedly Elevated After Induction of Colitis**

The synthesis of H₂S from endogenous substrate did not differ between samples that were taken from healthy rats and those taken from rats with colitis (Figure 1A). With samples from healthy controls, addition to the homogenate of exogenous L-cysteine did not significantly alter H₂S synthesis. However, when samples of inflamed colon were incubated with exogenous L-cysteine (Figure 1C), very large increases (>100-fold) in H₂S synthesis were observed. By 28 days after TNBS administration, when the colitis was largely resolved (Figure 1B), H₂S synthesis had returned to control levels (Figure 1C).

**CBS and CSE Contribute to Colonic H₂S Synthesis During Colitis**

The elevated capacity of inflamed colon to synthesize H₂S occurred via the enzymes CSE and CBS. The combination of inhibitors of these 2 enzymes reduced H₂S synthesis by healthy colonic tissue by ~50% (Figure 2), whereas that by colonic tissue from rats with colitis was reduced by 75%–98% by the 2 inhibitors, at all time points.

CBS appears to be the major source of H₂S synthesis in both healthy and inflamed colon (Figure 3). The CBS inhibitor (CHH) reduced H₂S synthesis by healthy colon by ~50%, whereas neither of the CSE inhibitors (BCA and PAG) had an effect. In the case of inflamed colon, the
CBS inhibitor reduced H₂S synthesis by approximately 75%.

**CSE and CBS Expression**

Despite the observation of a marked increase in the capacity of inflamed colon to synthesize H₂S, and the pharmacological evidence that this increase was largely due to synthesis via CBS, we observed an apparent down-regulation of the expression of CSE and CBS at some points when colonic injury and inflammation were substantial (Supplementary Figure 1). CBS expression was normal at 6 and 24 hours after TNBS administration, reduced at 3 and 7 days, and significantly elevated at 28 days. CSE expression was significantly lower at most time points. These data, together with the above-described evidence of a very large increase in the capacity of colonic tissue to generate H₂S from exogenous L-cysteine, suggest that changes in enzyme activity, rather than expression, accounted for the elevated H₂S production in this model of colitis.

Immunohistochemical staining of colonic tissue was not entirely consistent with the data from Western blotting. Expression of CBS was mainly evident in the muscularis mucosae and submucosa (Figure 4A–4C). As compared with controls (Figure 4A), CBS staining was diminished at 24 hours (Figure 4B) but more widespread at 7 days post-TNBS administration (Figure 4C). CSE staining was concentrated around blood vessels in the mucosa and submucosa (Figure 4D–4F). There was diminished staining at 24 hours (Figure 4E), but more extensive staining at 7 days post-TNBS administration (some of which appeared to be around sites of angiogenesis; Figure 4F). Goblet, crypt, and epithelial cells were immunonegative for CSE and CBS.

**Inhibition of H₂S Synthesis Exacerbates Colitis**

Having established that H₂S synthesis by the inflamed mucosa could be markedly reduced by treatment with a CBS inhibitor and to a lesser extent by 1 of the CSE inhibitors, we examined the effects on colitis of twice-daily administration of inhibitors over a 1-week period. As shown in Figure 5, there was substantial mortality observed in the groups of rats treated with each of the inhibitors, but particularly with CHH and PAG (note that PAG, unlike BCA, is an irreversible inhibitor of CSE²⁷). Indeed, none of the rats treated with CHH or PAG survived until the predefined end point of the study. In rats treated with BCA that survived until the end of the experiment, there was a significant increase in severity of colonic damage and in the thickness of the colon (Figure 6). The increase in the thickness of the colon in BCA-treated rats was mainly attributable to submucosal edema and hypertrophy of the muscularis (Figure 7A and 7B).

**Inhibition of H₂S Synthesis Triggers Colonic Inflammation**

It is possible that the high mortality observed in the rats with colitis that had been treated with CHH or PAG was due to exacerbation of colitis or perhaps unrelated to the colitis altogether. To determine whether suppression of H₂S synthesis in healthy rats resulted in death or elicited significant changes in the gastrointestinal tract, healthy rats were treated with CHH, PAG, or BCA (n = 4–6 per group) in the same manner as the rats with colitis had been treated. All rats treated with BCA twice daily for a week survived. Weight gain was the same in these groups (57 ± 2 g and 59 ± 4 g over 7 days, respectively). However, the small intestine and colon of the rats treated with BCA were inflamed and friable. Colonic MPO activity was significantly increased in BCA-
treated rats (4.9 ± 0.1 U/mg vs 3.6 ± 0.3 U/mg in vehicle-treated rats; P < .05). Treatment with BCA had no effect on colonic expression of mRNA for TNF-α (fold-change: 1.00 ± 0.08 in controls vs 1.25 ± 0.16 in BCA-treated), or COX-1 (fold-change: 1.00 ± 0.05 in controls vs 1.06 ± 0.05 in BCA-treated), but there was a significant decrease (>2-fold) in expression of COX-2 mRNA (fold-change: 1.00 ± 0.09 in controls vs −1.16 ± 0.13 with BCA; P < .05). A significant reduction (~50%) in colonic PGE₂ synthesis was also observed in the rats treated with BCA (45.1 ± 8.3 pg/mg vs 109.5 ± 11.1 pg/mg in healthy controls; P < .05). Treatment of healthy rats with PAG or CHH resulted in significant mortality (50% and 100%, respectively). In the case of CHH, the mortality in healthy rats occurred later (on days 3 or 4 of treatment) than that observed in rats with colitis (days 1 or 2 of treatment). In the rats that survived treatment with PAG, the small intestine and colon were hyperemic, atrophic, and friable. The mean colonic MPO activity was 6.3 U/mg (n = 2; the mean MPO in vehicle-treated rats was 3.6 ± 0.3 U/mg). As was the case with BCA, there was a marked down-regulation of cyclooxygenase (COX)-2 mRNA expression and prostaglandin E₂ synthesis (~50% of control levels) in the colon of surviving PAG-treated rats.

Role of ATP-sensitive K⁺ Channels

Treatment of rats with colitis with an antagonist of K⁺ₐₜₚ channels, glibenclamide, resulted in significant mortality (Figure 5). Within 2 days of initiating the twice-daily treatment with glibenclamide, 80% of the rats had died. In contrast, no mortality occurred in rats with colitis that were treated with pinacidil, a K⁺ₐₜₚ agonist. Pinacidil treatment did not affect the severity of colitis (after 1 week of treatment, damage scores of 13.4 ± 1.6 vs...
Healthy rats treated twice daily for a week with either pinacidil or glibenclamide were indistinguishable from vehicle-treated rats in terms of macroscopic appearance of the gastrointestinal tract. No deaths occurred, and the weight gain over a week of treatment with glibenclamide (55 ± 2 g) or pinacidil (60 ± 3 g) did not differ significantly from that of healthy rats treated with vehicle (59 ± 4 g).

**H₂S Donors Reduced the Severity of Colitis**

Twice-daily treatment by enema with either of 2 H₂S donors (NaHS and Lawesson’s reagent) resulted in a significant reduction in the severity of colitis (Figure 8). In addition to the colonic damage scores being significantly reduced as compared with the vehicle-treated group, both of the H₂S donors significantly attenuated the increase in colonic thickness that occurs in rats with colitis. However, there was no effect of the H₂S donors on MPO activity in the distal colon of the rats (vehicle: 129 ± 13 U/mg; NaHS: 87 ± 17 U/mg; Lawesson’s: 118 ± 9 U/mg), and histologic evaluation confirmed that there was extensive infiltration of the tissue with granulocytes (mainly neutrophils).

In vehicle-treated rats, there was a substantial up-regulation of the expression of TNF-α and COX-2 (∼7-fold and ∼30-fold over healthy controls, respectively) but no significant change in COX-1 expression. Treatment with either of the 2 H₂S donors significantly reduced TNF-α expression (Figure 8) but did not affect COX-1 or COX-2 expression. When colonic levels of TNF-α protein were examined, a significant reduction (50%) was observed in the group treated with NaHS (12.6 ± 3.5 pg/mg protein vs 6.5 ± 1.6 pg/mg protein, respectively; \( P < .05 \)). In the group treated with Lawesson’s reagent, there was considerable variability in the data, with no significant difference from the control group.

**Discussion**

H₂S is produced throughout the body, and there is growing evidence that it plays an important role in numerous physiological and pathophysiological processes. In the present study, we have observed that H₂S synthesis by the colon of the rat increases dramatically (∼100-fold) after induction of colitis, returning to control levels as the inflammation resolves. The primary enzymatic source
of H$_2$S in this context is CBS, but CSE also makes an important contribution. Inhibition of H$_2$S synthesis resulted in exacerbation of colitis and to significant mortality (which does not appear to be entirely attributed to effects of the inhibitors on the colon). Moreover, inhibition of H$_2$S synthesis in healthy rats led to significant intestinal inflammation and loss of mucosal integrity, as well as a down-regulation of expression of COX-2 mRNA. Administration of either of 2 H$_2$S donors significantly reduced the severity of colitis, as well as mucosal TNF-α mRNA expression. Taken together, these findings suggest that H$_2$S contributes to the resolution of colitis and is an endogenous antiinflammatory mediator in the colon.

Schicho et al demonstrated expression of CSE and CBS in the enteric nervous system of the guinea pig and human intestine. In the present study, we detected expression of both of these enzymes in the rat colon; CBS staining was not apparent in the epithelium but was in the lamina propria consistent with the observations with Schicho et al. The staining for CSE was quite diffuse, consistent with previous reports that this enzyme is expressed within vascular elements, as well as with the report of Schicho et al that CSE is expressed in over 90% of submucous and myenteric nerves. It is interesting that CBS was, at least based on the effects of inhibitors, the major source of colonic H$_2$S, both in the healthy state and when inflamed.

H$_2$S produces a wide variety of effects that may contribute to its ability to modulate colitis. It can inhibit leukocyte adherence to the endothelium and reduce edema formation; suppress the expression of several proinflammatory cytokines and enzymes, such as TNF-α, IFN-γ, and iNOS; act as an antioxidant; and promote healing. At least some of these effects may be related to inhibition of activation of NF-κB. In the present study, we observed a significant down-regulation of expression of mRNA for TNF-α in rats with colitis treated with H$_2$S donors (and in the case of NaHS, a significant reduction of TNF-α protein in the colon). Previously, a garlic-derived substance that releases H$_2$S (allyl trisulfide) was shown to suppress TNF-α expression and NF-κB activation in colonic biopsy samples from ulcerative colitis patients. We also observed that an inhibitor of H$_2$S synthesis markedly down-regulated colonic COX-2 activity, and this was accompanied by reduced colonic prostaglandin E$_2$ synthesis and induction of inflammation in the tissue. COX-2 is a source of several antiinflammatory eicosanoids and plays a crucial role in the resolution of inflammation in many tissues, including the gastrointestinal tract. Indeed, COX-2 plays a crucial role in the resolution of TNBS-induced colitis in rats, including being a source of important antiinflammatory mediators (eg, prostaglandin D$_2$).

Several actions of H$_2$S, including vasodilation, inhibition of leukocyte adherence, and visceral analgesia, have...
been suggested to be mediated via activation of \(K_{\text{ATP}}\) channels.\(^{3,5,31}\) In the present study, administration of pinacidil, an agonist of \(K_{\text{ATP}}\) channels, did not confer any beneficial effects. On the other hand, treatment with glibenclamide, an antagonist of \(K_{\text{ATP}}\) channels, led to significant mortality in rats with colitis. This appears to have been due to a worsening of the colitis, rather than a nonspecific toxicity of glibenclamide, because no mortality or weight loss was observed when healthy rats were similarly treated. Thus, it is possible that activation of \(K_{\text{ATP}}\) channels by endogenous \(H_2S\) contributes to its ability to attenuate the severity of colitis.

The question of whether \(H_2S\) is beneficial vs detrimental to the colon has been controversial for many years. Some have suggested that the \(H_2S\) produced in the lumen of the colon may contribute to the pathogenesis of ulcerative colitis and/or colon cancer.\(^{12-15}\) There certainly appears to be at least as much data countering this claim as there are supporting it, and questions have been raised as to the accuracy of some estimates of very high concentrations of \(H_2S\) in the lumen.\(^{16-18,44,45}\) Most of the \(H_2S\) that is produced in the lumen of the intestine is absorbed and rapidly metabolized.\(^{46}\) Indeed, efficient detoxification occurs via a number of enzymes present in the mucosa,\(^{17}\) and no impairment of these detoxification systems was detected in patients with ulcerative colitis or Crohn’s disease. Based on studies utilizing a rat model of colitis (dextran sodium sulfate), Furne et al\(^{47}\) concluded that “excessive \(H_2S\) production” did not contribute to tissue injury. The results from the present study point to a predominantly positive role for endogenous \(H_2S\) (ie, that produced by the colon itself). Moreover, a garlic-derived product (garlicin) that can release \(H_2S\) was shown to reduce the severity of TNBS-induced colitis in rats.\(^{48}\)

An increase in colonic \(H_2S\) synthesis in samples of inflamed colon was detectable only when exogenous L-cysteine was added to the reaction mixture. This raises the possibility that tissue L-cysteine levels may “regulate” colonic \(H_2S\) synthesis, at least in the context of inflammation. However, this requires further investigation.

In summary, the present studies provide evidence that both endogenous and exogenous \(H_2S\) can promote the resolution of experimental colitis. The contribution of bacterially derived \(H_2S\) in a setting of colitis remains unclear. These findings may have important implications for the therapy of IBD.

**Supplementary Data**

Note: To access the supplementary material accompanying this article, visit the online version of

References


Received December 5, 2008. Accepted April 9, 2009.

Reprint requests
Address requests for reprints to: John L. Wallace, PhD, Department of Medicine, McMaster University, 1200 Main Street West, Hamilton, Ontario, L8N 3Z5, Canada. e-mail: jwalla@mcmaster.ca; Fax: (905) 528-9862.

Conflicts of interest
The authors disclose no conflicts.

Funding
Supported by a grant from the Canadian Institutes of Health Research (CIHR), by a Canadian Association of Gastroenterology/AstraZeneca Canada/CIHR Fellowship (to G.R.M.).
Supplementary Figure 1. Western blots of cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) in healthy (Cont) and inflamed colon of rats. Panel A shows examples of blots, whereas panels B and C show the densitometric data on expression of CSE and CBS, respectively (normalized to β-actin expression). *P < .05 vs the control group. Each group consisted of 3 or 4 rats.