

Hydrogen sulfide enhances ulcer healing in rats

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ABSTRACT Hydrogen sulfide is an endogenous mediator that relaxes vascular smooth muscle, exhibits several antiinflammatory activities, and contributes to gastric mucosal defense. This study was performed to examine the role of hydrogen sulfide in the resolution of injury; specifically, the healing of gastric ulcers. Ulcers were induced in rats by serosal application of acetic acid. This elicited a marked increase in gastric expression of the two key enzymes in hydrogen sulfide synthesis (cystathionine- β -synthase and cystathionine- γ -lyase) and in hydrogen sulfide synthesis. Twice-daily treatment for a week with hydrogen sulfide donors significantly increased the extent of healing of gastric ulcers as compared to vehicle-treatment. Similar treatment with L-cysteine, a precursor for hydrogen sulfide, also accelerated healing of the ulcers, and the effect was abolished by cotreatment with an inhibitor of cystathionine- γ -lyase. The beneficial effects of hydrogen sulfide on ulcer healing were not dependent on nitric oxide synthesis, nor did they appear to occur through activation of ATP-sensitive K⁺ channels. These results suggest that hydrogen sulfide is produced in the gastric mucosa in response to injury and acts to promote healing. The results further suggest that drugs releasing hydrogen sulfide could be employed to accelerate healing of gastric ulcers, and possibly of other wounds.—Wallace, J. L., Dicay, M., McKnight, W., Martin, G. R. Hydrogen sulfide enhances ulcer healing in rats. *FASEB J.* 21, 4070–4076 (2007)

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GASTRIC ULCERATION ASSOCIATED with *Helicobacter pylori* infection is becoming less of a significant clinical issue in many developed countries as the rate of infection with this bacterium declines. However, ulcers associated with the use of nonsteroidal antiinflammatory drugs (NSAIDs) continue to be a major clinical problem, even after the introduction of selective inhibitors of cyclooxygenase-2. Although agents that profoundly suppress gastric acid secretion are very effective in promoting the healing of ulcers (1), ulcer bleeding and recurrence remain a significant problem.

A number of endogenous substances have been shown to contribute to the healing of gastric ulcers, including prostaglandins (PG), epidermal growth factor, fibroblast growth factor, and nitric oxide (NO) (2–6). Recently, a role for another gaseous mediator in mucosal defense has been suggested. Hydrogen sulfide is a vasodilator and neuromodulator (7) and contributes to the resistance of

the gastric mucosa to injury (8, 9). NSAIDs reduce endogenous H₂S synthesis, while administration of H₂S donors can reduce the severity of NSAID-induced gastric damage in rats (8). Moreover, an H₂S-releasing NSAID derivative was recently shown to produce substantially less gastrointestinal injury than the parent NSAID (diclofenac), despite still suppressing gastric prostaglandin synthesis (10). In addition to contributing to mucosal resistance to injury, H₂S shares with nitric oxide the ability to inhibit leukocyte adherence to the vascular endothelium (8, 11), as well as analgesic effects (12, 13).

Given the role of H₂S in mucosal defense, and its similar vasoactive and antiinflammatory activities to NO, we investigated the possibility that H₂S contributes to the healing of experimental gastric ulcers. This involved an investigation of the effects of endogenous H₂S and of exogenous H₂S donors. We also examined whether or not H₂S could counteract the detrimental effects of an NSAID on gastric ulcer healing. Finally, we examined several potential mechanisms through which H₂S may modulate ulcer healing. Our results suggest that, like nitric oxide, H₂S makes a significant contribution to the healing of ulcers, and H₂S donors may have some utility in terms of promoting healing of ulcers and wounds. These effects may be related to the vasodilator actions of H₂S but are not mediated via K⁺_{ATP} channel- or NO-dependent pathways.

MATERIALS AND METHODS

Animals

Male Wistar rats (175–200 g) were obtained from Charles River (Montreal, QC, Canada). They were fed standard laboratory chow and tap water and were kept in a room with controlled temperature (22±1°C), humidity (65–70%), and light cycle (12-h light/12-h dark). All experiments were approved by the University of Calgary Animal Care Committee.

Ulcer induction

The rats were fasted for 18 h. Under halothane anesthesia, acetic acid (0.5 ml, 80% v/v) was applied to the serosal surface of the stomach for 1 min *via* a 3 ml syringe barrel (6, 14). The abdomen was sutured closed, and the rats were returned to their cages. On day 10 after ulcer induction, rats were euthanized with sodium pentobarbital. The stomach then was removed and the ulcer area was measured planimetrically in a blind manner (6).

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Treatment regimens

Groups of at least 5 rats each were given vehicle (0.5% carboxymethylcellulose; 1 ml/kg), or one of several test drugs intragastrically twice each day from day 3 to day 9 after ulcer induction. The test drugs included the hydrogen sulfide donors, Lawesson's reagent and 4-hydroxythiobenzamide (4HTB), at 10 or 30 $\mu\text{mol/kg}$, and the hydrogen sulfide precursor, L-cysteine (15, 50, or 100 mg/kg). We also assessed the effects of a nonsteroidal antiinflammatory drug (diclofenac, at 5 mg/kg), alone or in combination with Lawesson's reagent (30 $\mu\text{mol/kg}$). The effects on ulcer healing of a hydrogen sulfide synthesis inhibitor, propargylglycine (PAG; 50 mg/kg i.p.; inhibitor of CSE), given alone and together with L-cysteine (50 mg/kg), were also assessed.

As a positive control, we also assessed the effects of a nitric oxide donor (glyceryl trinitrate; GTN) at 1 mg/kg, since it has previously been shown to accelerate healing in this model (6). GTN was tested alone, and in combination with an H_2S donor (Lawesson's reagent, 30 $\mu\text{mol/kg}$).

Effects of H_2S on gastric eicosanoid synthesis

Groups of rats ($n=4-9$) with ulcers and of healthy controls were treated twice-daily with Lawesson's reagent (30 $\mu\text{mol/kg}$), 4HTB (30 $\mu\text{mol/kg}$), or vehicle each day, beginning on day 3 after ulcer induction and continuing for 4 days. Two hours after the final administration of the H_2S donor or vehicle, the rats were killed and samples of the gastric mucosa (from the region of the ulcer margin or the corresponding region in controls) were excised and processed, as described previously (15) for measurement of PGE_2 and leukotriene (LT) B_4 synthesis. Other samples of the gastric tissue were processed for blind histological analysis (hematoxylin and eosin staining).

Role of nitric oxide and K^+_{ATP} channels

To determine whether the effects of an H_2S donor on gastric ulcer healing occurred in an NO-dependent manner, we examined the effects of treatment with a nonselective nitric oxide synthase inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME; 15 mg/kg twice-daily) in rats receiving vehicle or Lawesson's reagent (30 $\mu\text{mol/kg}$). As in the other experiments, treatments were carried out from day 3 to day 10 postulcer induction.

Some actions of H_2S have been shown to be mediated *via* activation of K^+_{ATP} channels (11, 16). To determine if such actions might contribute to the effects of H_2S on ulcer healing, experiments were performed in which glibenclamide, a K^+_{ATP} channel antagonist, was administered orally twice-daily (10 mg/kg) from days 3 to 10 after ulcer induction. In other experiments, the a K^+_{ATP} channel agonist, pinacidil (10 mg/kg), was administered in the same manner.

Expression of CSE and CBS

Cystathionine- β -synthase (CBS) and cystathionine- γ -lyase (CSE) are the principal enzymes involved in the generation of H_2S from cysteine, cystine, and homocysteine. Western blot analysis of gastric tissue CSE and CBS levels was performed on either healthy rats or in rats 1, 3, and 6 days following the induction of ulcers. Rat stomachs were isolated, opened from the pylorus along the greater curvature, and pinned out in iced saline. Following surface area measurements, half of the ulcer, including the surrounding ulcer margin, was quickly isolated, snap-frozen, and stored at -80°C until analysis. The frozen samples were homogenized in ice-cold lysis buffer composed of 20 mmol/L Tris-HCl, 0.1 mmol/L PMSF, and 5 $\mu\text{l/ml}$ protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA contain-

ing 4-[2-aminoethyl]benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin). The protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA), and then an aliquot of the lysate was placed into Laemmli sample buffer and boiled for 2 min. Equal amounts of protein (40 μg) were loaded onto a 10% gel, subjected to SDS-PAGE, and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Hybond-P PVDF Membrane, Amersham Biosciences, Buckinghamshire, UK). CSE and CBS were detected using a 1:1000 dilution (24 h at 4°C) of mouse monoclonal antibody to either cystathionine γ -lyase or cystathionine β -synthase (Abnova, Taipei, Taiwan). The membrane was then incubated with HRP-conjugated anti-mouse IgG (1:4000) and detected by enhanced chemiluminescence on HyperfilmTM ECL film (Amersham Biosciences, Piscataway, NJ, USA). Band density (INT/ mm^2) was determined using a calibrated imaging densitometer (GS-710, Bio-Rad) and Quantity One software (Bio-Rad, Hercules, CA, USA).

Gastric H_2S synthesis

Synthesis of H_2S by gastric tissue was measured using the method of Abe & Kimura (17), as modified by Qu *et al.* (18). Groups of 5 rats each were killed 1 to 9 days after ulcer induction; their stomachs were quickly removed and opened from the pylorus along the greater curvature and then pinned out in iced saline. Half of the ulcer, including the surrounding ulcer margin, was quickly isolated, snap-frozen, and stored at -80°C . The gastric tissue was homogenized in ice-cold 50 mmol/L potassium phosphate buffer, pH 8.0 (12% w/v), with a Polytron homogenizer (Brinkmann Instruments, Rexdale, ON, Canada). The homogenate (0.5 ml) and buffer (0.4 ml) were then cooled on ice for 10 min before L-cysteine (10 mmol/L) and pyridoxal 5'-phosphate (2 mmol/l) were added. The final volume was 1 ml. A smaller 2-ml tube containing a piece of filter paper (0.5 \times 1.5 cm) soaked with zinc acetate (1%; 0.3 ml) was put inside the larger vial. The vials were then flushed with nitrogen gas for 20 s and capped with an airtight serum cap. The vials were then transferred to a 37°C shaking water bath and, after 90 min, trichloroacetic acid (TCA; 50%; 0.5 ml) was injected into the reaction mixture through the serum cap. The mixture was left to stand for another 60 min to allow for the trapping of evolved H_2S by the Zn acetate. The serum cap was then removed and N, N-dimethyl-p-phenylenediamine sulfate (20 mmol/L; 50 μl) in 7.2 mol/L HCl and FeCl_3 (30 mmol/L; 50 μl) in 1.2 mol/L HCl were added to the inner tube. After 20 min, absorbance at 670 nm was measured with a microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA). The calibration curve of absorbance *vs.* H_2S concentration was obtained by using NaHS solution of varying concentrations as described previously (18).

Acid secretion

Groups of 5 rats each were given vehicle, Lawesson's reagent, or 4HTB (the drugs at 30 $\mu\text{mol/kg}$) orally. After 15 min, they were anesthetized with Halothane, the abdomen was opened, and the pyloric sphincter was ligated. The abdominal wound was closed with sutures, and the rats were left for 3 h. They were then reanesthetized, and the stomach was removed, with care taken to preserve the gastric contents. The volume of the gastric contents and pH were determined.

Tissue glutathione levels

Glutathione is an endogenous antioxidant that can be synthesized from L-cysteine and has been reported to contribute to gastric mucosal defense (19). To determine whether beneficial

effects of H₂S may be mediated by induction of glutathione in gastric tissue, experiments were performed in which glutathione levels were measured after oral treatment with L-cysteine at doses that promoted ulcer healing. Groups of 5 rats were given vehicle or L-cysteine (15 or 100 mg/kg). After 3 h, the rats were euthanized and samples of the stomach and liver were excised and processed measurement of tissue glutathione levels, using a commercially available kit (Cayman Chemical, Ann Arbor, MI, USA). This assay uses an optimized enzymatic recycling method, using glutathione reductase for the quantification of GSH. Both GSH and GSSG are measured, so the assay reflects total glutathione. As a negative control, a group of rats was treated orally with diethyl maleate (0.6 ml/kg), which has been reported to deplete tissue glutathione (20).

Statistical analysis

All data are expressed as mean ± SEM, with sample sizes of at least 5 per group. Comparisons of data among groups were performed with one-way ANOVA followed by the Student–Newman–Keuls test. An associated probability (*P*-value) of less than 5% was considered significant.

Materials

Lawesson’s reagent, PAG, L-cysteine, glibenclamide, diethyl maleate, 5-ASA, and pinacidil were obtained from Sigma-Aldrich. 4HTB (4-hydroxythiobenzamide) was obtained from SynChem (Des Plaines, IL, USA). ATB-429 (5-amino-2-hydroxy-benzoic acid 4-(5-thioxo-5H-[1,2]dithiol-3-yl)-phenyl ester hydrochloride) was provided by Antibe Therapeutics Inc. (Toronto, ON, Canada). All other reagents, unless otherwise specified, were obtained from Fisher (Edmonton, AB, Canada).

RESULTS

Hydrogen sulfide synthesis is increased follow ulcer induction

Induction an ulcer in the rat stomach resulted in a marked increase in gastric H₂S synthesis, as shown in Fig. 1. H₂S synthesis was not significantly elevated 1 day after ulcer induction but was at days 3 and 5. It declined thereafter to levels not significantly different from those in controls.

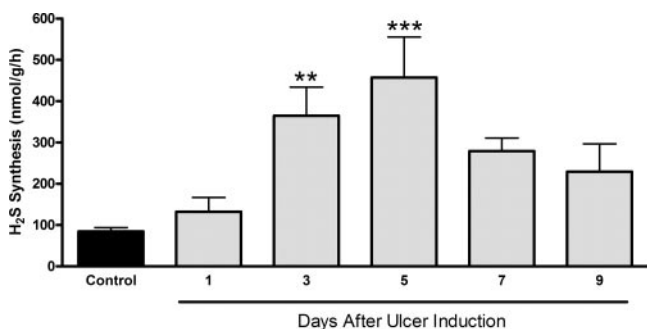


Figure 1. Effects of ulceration on hydrogen sulfide synthesis by gastric tissue. The ability of gastric tissue to synthesize H₂S was assessed in healthy control rats and in rats at various times after induction of a gastric ulcer. Each bar represents the mean ± SEM of 3–6 samples. ***P* < 0.01, ****P* < 0.001 *vs.* the control group.

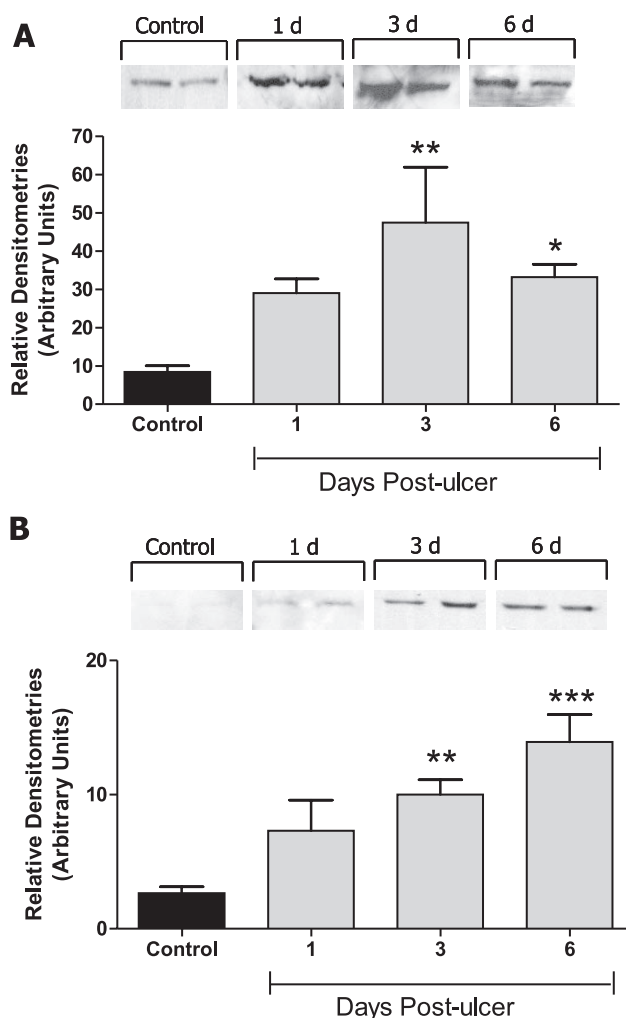


Figure 2. Effects of gastric ulceration on expression of two key enzymes in hydrogen sulfide synthesis, cystathionine-γ-lyase (CSE; A) and cystathionine-β-synthase (CBS; B). Western blot analysis of gastric tissue CSE and CBS expression was performed on tissue from healthy rats or rats with ulcers (1, 3, and 6 days following ulcer induction). A representative Western blot is shown above each graph (2 samples per time point). In the histogram, each bar represents the mean ± SEM for 5 samples. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *vs.* the corresponding control group.

Both of the key enzymes for H₂S synthesis, CSE, and CBS, were constitutively expressed in the stomach of healthy rats (Fig. 2). Following induction of an ulcer, there was a marked increase in the expression of both enzymes. This was seen in samples taken at both 3 and 6 days after ulcer induction.

Exogenous H₂S donors enhanced ulcer healing

Gastric ulcers healed significantly during the period between day 3 and day 10 after their induction (*i.e.*, in rats treated with vehicle) (Fig. 3). Twice-daily treatment with the H₂S donors, Lawesson’s reagent or 4HTB, at 30 μmol/kg resulted in significantly greater healing (*i.e.*, the ulcers on day 10 were significantly smaller than those in the vehicle-treated group). The lower dose of

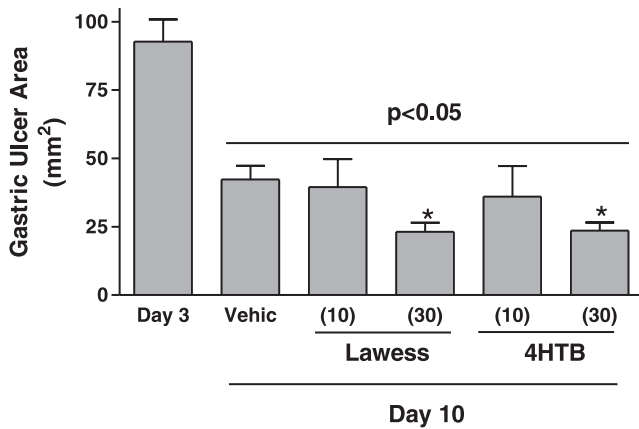


Figure 3. Hydrogen sulfide donors promote the healing of gastric ulcers in rats. Twice-daily treatment with either Lawesson's reagent or 4HTB, at 30 $\mu\text{mol/kg}$ (but not at 10 $\mu\text{mol/kg}$), significantly improved healing as compared to treatment with vehicle ($*P<0.05$). Treatment was initiated on day 3 and continued for a week. Significant healing occurred in all groups during the treatment period (*i.e.*, as compared to the "Day 3" group). Each bar represents the mean \pm SEM of at least 5 rats.

Lawesson's reagent and 4HTB (10 $\mu\text{mol/kg}$) did not significantly affect ulcer healing as compared to the vehicle-treated group.

Blind histological examination of the tissues from rats treated with Lawesson's reagent (30 $\mu\text{mol/kg}$) or vehicle failed to reveal any obvious phenotypic changes in the ulcer bed. Substantial infiltration of granulocytes (primarily neutrophils) in the ulcer bed was apparent in both groups.

Nitric oxide donors have previously been reported to accelerate gastric ulcer healing in rats (2, 6). To determine whether an H_2S donor and an NO donor could produce enhanced gastric ulcer healing *vs.* either agent alone, we tested the effects of twice-daily treatment with glyceryl trinitrate (GTN), Lawesson's reagent, or both. As shown in **Fig. 4**, both the NO donor and the H_2S donor significantly improved healing as compared to the vehicle-treated group. The combination of the NO donor and H_2S donor produced even greater healing; that is, the ulcers were significantly smaller with the combination therapy than with either of the donors alone.

Endogenous NO did not appear to contribute to the promotion of ulcer healing by H_2S . Pretreatment with L-NAME did not block the enhancement of healing observed in rats treated with Lawesson's reagent (mean ulcer area of $26.6\pm 6.4 \text{ mm}^2$ *vs.* $23.4\pm 3.7 \text{ mm}^2$ in the group treated with Lawesson's reagent alone).

PGE_2 synthesis by gastric tissue from rats with ulcers (day 7 after ulcer induction) was elevated ~ 9 -fold over that by gastric tissue from healthy controls ($622\pm 100 \text{ ng/mg}$ *vs.* $68\pm 17 \text{ ng/mg}$, respectively; $P<0.05$). Similarly, the synthesis of LTB_4 by gastric tissue from rats with ulcers was elevated ~ 7 -fold over that by gastric tissue from healthy controls ($5.4\pm 0.9 \text{ ng/mg}$ *vs.* $0.8\pm 0.2 \text{ ng/mg}$, respectively; $P<0.05$). In rats with ulcers, twice-daily treatment for 4 days with Lawesson's reagent or 4HTB (both at 30 $\mu\text{mol/kg}$) did not significantly change gastric PGE_2 synthesis ($445\pm 85 \text{ ng/mg}$ and 509 ± 60 , respectively) or

LTB_4 synthesis ($4.2\pm 1.3 \text{ ng/mg}$ and 7.1 ± 2.0 , respectively). Similarly, treating healthy controls twice-daily with Lawesson's reagent or 4HTB had no effect on PGE_2 ($29\pm 11 \text{ ng/mg}$ and $47\pm 21 \text{ ng/mg}$, respectively) or LTB_4 ($0.8\pm 0.2 \text{ ng/mg}$ and $0.6\pm 0.1 \text{ ng/mg}$, respectively) synthesis.

A hydrogen sulfide precursor enhanced ulcer healing

Endogenous H_2S synthesis *in vivo* can be boosted by administration of the precursor amino acid, L-cysteine (18). We found that twice-daily administration of L-cysteine significantly enhanced gastric ulcer healing as compared to vehicle (**Fig. 5**). Administration of an inhibitor of endogenous H_2S synthesis, PAG, which blocks CSE activity, impaired ulcer healing. L-cysteine failed to significantly improve healing when given in combination with the inhibitor of CSE. Thus, endogenous H_2S appears to make an important contribution to the healing of gastric damage, consistent with a previously reported role of H_2S as a mediator of gastric mucosal defense (8).

The possibility that the beneficial effects of H_2S on ulcer healing may be mediated *via* activation of K^+_{ATP} channels was examined by testing the effects of an agonist of this channel (pinacidil). While we have previously observed that pinacidil mimicked the antiinflammatory effects of H_2S , it did not produce a beneficial effect on ulcer healing as was observed with H_2S donors (mean ulcer area in pinacidil-treated rats was $34.2\pm 15.2 \text{ mm}^2$, as compared to $39.7\pm 4.2 \text{ mm}^2$ with vehicle). Moreover, treating rats twice-daily with glibenclamide, an antagonist of the K^+_{ATP} channel, did not significantly affect ulcer healing (mean ulcer area of $34.1\pm 3.9 \text{ mm}^2$).

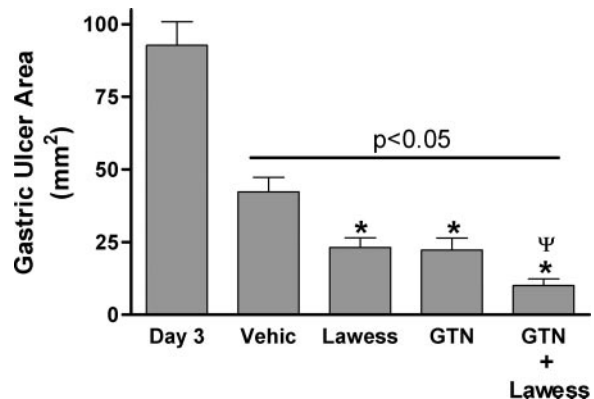


Figure 4. Additive benefit on ulcer healing of combined administration of a hydrogen sulfide donor (Lawesson's reagent) and a nitric oxide donor (GTN; glyceryl trinitrate). On day 3 after ulcer induction, twice-daily treatment with vehicle, Lawesson's reagent (30 $\mu\text{mol/kg}$), GTN (1 mg/kg), or both Lawesson's reagent and GTN was initiated. After one week of treatment, ulcer areas were measured. There was significant healing in all groups during the week of treatment. Lawesson's reagent and GTN significantly improved healing ($*P<0.05$ *vs.* vehicle), the combination of the two reduced ulcer area significantly below that with either drug alone ($^{\Psi}P<0.05$). Bars represent the mean \pm SEM, with at least 5 rats.

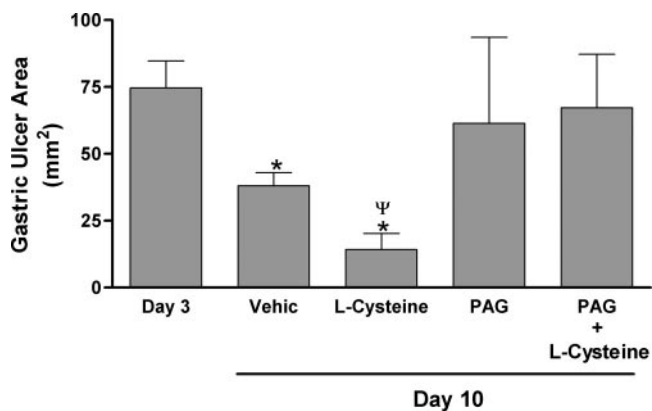


Figure 5. Enhanced healing of gastric ulcers in rats by twice-daily administration of L-cysteine (50 mg/kg). Ulcers in rats treated with vehicle healed significantly during the period between day 3 and day 10 after their induction ($*P < 0.05$ vs. day 3). Ulcers in L-cysteine-treated rats were significantly ($^{\psi}P < 0.05$) smaller on day 10 than those in vehicle-treated rats. Rats given the CSE inhibitor propargylglycine (PAG; 50 mg/kg i.p.) did not exhibit significant healing of ulcers at day 10 (vs. day 3). Moreover, the beneficial effects of L-cysteine administration were not seen in rats coadministered PAG. Bars represent the mean \pm SEM, with at least 5 rats.

NSAID-induced delayed ulcer healing is reversed by a hydrogen sulfide donor

Nonsteroidal antiinflammatory drugs (NSAIDs) can retard healing of gastric ulcers in humans and in animal models (6, 21, 22). Indeed, treatment of rheumatic conditions in patients with preexisting ulcers is a significant clinical problem. We examined the possibility that an H₂S donor would abrogate the detrimental effects of an NSAID on ulcer healing. As shown in Fig. 6, twice-daily administration of diclofenac resulted in impaired ulcer healing relative to vehicle-treated rats. In rats cotreated with diclofenac and Lawesson's reagent, significant healing of the ulcers was apparent (although not to the same extent seen with Lawesson's reagent alone).

An H₂S-releasing salicylate derivative enhanced ulcer healing

Lawesson's reagent and 4HTB are commercially available H₂S donors. We also evaluated the effects of a proprietary antiinflammatory drug which releases H₂S, namely ATB-429. This drug is an H₂S-releasing derivative of 5-aminosalicylic acid (5-ASA), which has been shown to have enhanced antiinflammatory activity as compared to the parent drug (23). As shown in Fig. 6, treatment with 5-ASA did not significantly affect ulcer healing as compared to vehicle. However, treatment with an equimolar dose of ATB-429 resulted in a significant improvement in ulcer healing as compared to the vehicle-treated group.

H₂S donors do not affect gastric acid secretion

Treatment with either Lawesson's reagent or 4HTB (each at 30 μ mol/kg) did not significantly affect gastric

acid secretion as compared to vehicle treatment. The mean volume of gastric juice produced over a 3-h period in vehicle-, Lawesson's reagent-, and 4HTB-treated rats was 3.7 ± 1.1 , 3.2 ± 0.4 , and 3.1 ± 0.6 ml, respectively, while the pH was 1.35 ± 0.06 , 1.42 ± 0.04 , and 1.49 ± 0.05 , respectively.

Changes in gastric glutathione do not contribute to prohealing effects of H₂S

Basal levels of glutathione were greater in liver than in gastric tissue. Administration of a high dose of L-cysteine (100 mg/kg) resulted in a significant (~ 10 -fold) increase in glutathione levels in the liver, but not the stomach (Fig. 7). This dose of L-cysteine reduced ulcer area significantly as compared to vehicle treatment (9.5 ± 5.3 mm² vs. 38.0 ± 4.9 mm², respectively; $P < 0.01$). Treatment with diethyl maleate resulted in significant reductions in glutathione levels in both tissues. When L-cysteine was administered at 15 mg/kg, a dose that also significantly accelerate gastric ulcer healing (20.9 ± 5.5 mm²; $P < 0.05$ vs. vehicle-treated), it did not affect glutathione levels in the stomach or liver.

DISCUSSION

While long recognized as an industrial pollutant, hydrogen sulfide is increasingly identified as an important mediator of many physiological processes. In addition to roles as a neuromodulator and vasodilator (7, 17), H₂S has been shown to be an endogenous regulator of acute inflammation (11) and pain (13) and to contribute to the maintenance of gastric mucosal integrity (8). The present study focused on the role of H₂S in the resolution of

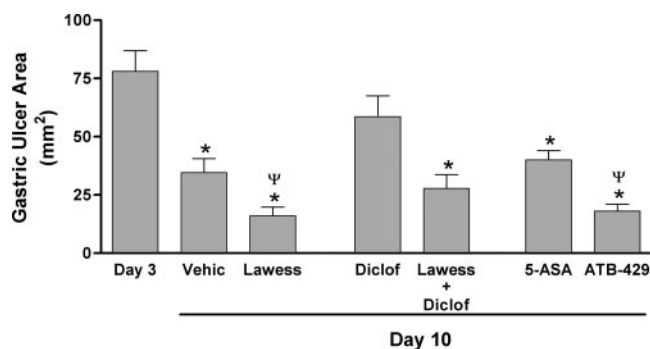


Figure 6. Enhanced healing of gastric ulcers in rats by twice-daily administration of L-cysteine. Ulcers in rats treated with vehicle healed significantly during the period between day 3 and day 10 after their induction ($*P < 0.05$ vs. day 3). Ulcers in rats treated with diclofenac were not significantly different in area than those in the rats at day 3. However, coadministration of Lawesson's reagent (30 μ mol/kg), which itself enhanced healing vs. vehicle treatment ($^{\psi}P < 0.05$), restored healing in rats treated with diclofenac (5 mg/kg). Treatment with 5-aminosalicylic acid (5-ASA; 50 mg/kg) did not significantly affect ulcer healing vs. vehicle. However, treatment with an equimolar dose of ATB-429, an H₂S-releasing derivative of 5-ASA, significantly enhanced ulcer healing as compared to vehicle and to 5-ASA.

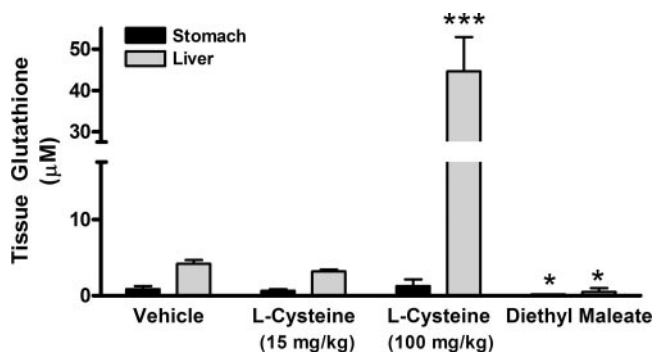


Figure 7. Tissue glutathione levels in rats treated with L-cysteine or diethyl maleate. Rats were treated orally with vehicle, L-cysteine or diethyl maleate (0.6 ml/kg), and gastric tissue was harvested for measurement of glutathione levels 3 h later (see Materials and Methods for details of assay procedure). L-cysteine was tested at two doses (15 and 100 mg/kg), both of which enhanced ulcer healing. Diethyl maleate was used as a further control for this assay, as it depletes tissue glutathione levels. Bars represent the mean \pm SEM of at least 5 rats. * $P < 0.05$, *** $P < 0.001$ vs. the corresponding vehicle-treated group.

injury; specifically, gastric ulcer healing. We found that H_2S is produced by the healthy gastric mucosa, and both of the key enzymes for mammalian H_2S synthesis, CSE and CBS, are constitutively expressed in the mucosa. Induction of gastric ulceration was associated with a profound increase in the expression of these two enzymes, and of H_2S synthesis. Our findings suggest that this may be a defensive response, aimed at mitigating tissue injury and accelerating repair. Indeed, enhanced ulcer healing was observed following administration of three chemically distinct hydrogen sulfide donors. Moreover, administration of L-cysteine, a precursor of endogenous H_2S synthesis, resulted in enhanced healing. This beneficial effect of L-cysteine was prevented by coadministration of an inhibitor of CSE, strongly suggesting that the effect of L-cysteine was mediated *via* H_2S production. Taken together, these results therefore suggest an important role for H_2S in the process of healing of chronic gastric ulcers.

Several possible mechanisms for the acceleration of ulcer healing by H_2S were considered. Profound suppression of gastric acid secretion, as can be achieved by administration of proton pump inhibitors, will accelerate gastric and duodenal ulcer healing (1). It was possible that the enhancement of ulcer healing by H_2S donors might have also occurred through inhibition of acid secretion, or by otherwise raising gastric pH. However, we found this not to be the case. Neither 4HTB nor Lawesson's reagent, at doses that accelerated ulcer healing, had an effect on the volume or the pH of gastric juice.

We next investigated the possible role of K^+_{ATP} channels in the ulcer healing process. Many actions of H_2S , including vasodilation, inhibition of leukocyte adherence, visceral analgesia, and inhibition of edema formation, have been found to be inhibited by a K^+_{ATP} channel antagonist (glibenclamide) and mimicked by a K^+_{ATP} channel agonist (pinacidil) (11, 13, 16). However, we observed that neither glibenclamide nor pinacidil significantly affected ulcer healing.

Nitric oxide and H_2S share many similar actions, including an ability to contribute to the healing of ulcers. There is evidence that H_2S can enhance the ability of NO to relax smooth muscle (24). H_2S can also promote the release of NO from the vascular endothelium (which may contribute to the above-mentioned effect). On the other hand, NO can regulate the synthesis of H_2S (25), at least in part by increasing the expression and activity of CSE (7, 26). These observations raised the possibility that the enhancement of ulcer healing induced by H_2S in the present study may have occurred in an NO-dependent manner. However, pretreatment with the nonselective NOS inhibitor, L-NAME, did not diminish the ability of an H_2S donor (Lawesson's reagent) to enhance ulcer healing.

Agents containing sulfhydryl groups have been shown to be protective in a number of models of acute gastric injury (18, 27), but the mechanism underlying this action is unclear. An influence on gastric levels of the antioxidant substance, glutathione, has been suggested as a possible mechanism (19, 20). Indeed, there are links between the synthesis of glutathione and the synthesis of H_2S , since L-cysteine is a common substrate for both. We, therefore, examined the possibility that the beneficial effects of L-cysteine on ulcer healing may have been due to increases in gastric glutathione levels, rather than to effects on H_2S synthesis. First, we observed that administration of an inhibitor of CSE, propargylglycine (PAG), prevented the enhancement of ulcer healing by L-cysteine. Indeed, PAG itself significantly impaired ulcer healing, consistent with a contribution of endogenous H_2S to the healing process. Second, administration of L-cysteine, even at a dose well above that which significantly accelerated ulcer healing, did not affect gastric levels of glutathione. This high dose of L-cysteine (100 mg/kg) could induce a ~ 10 -fold increase in hepatic glutathione levels. On the other hand, a lower dose of L-cysteine, which still enhanced ulcer healing, had no effect on gastric or hepatic glutathione levels. While we cannot rule out a contribution of glutathione to the ulcer healing process, these observations are consistent with the hypothesis that the effects of L-cysteine on ulcer healing were attributable to augmented H_2S generation. It is noteworthy that L-cysteine has previously been reported to enhance ulcer healing in a clinical setting. Salim (27) reported that treatment with L-cysteine together with cimetidine significantly enhanced the healing of duodenal ulcers as compared to treatment with cimetidine alone. Moreover, the treatment that included L-cysteine was associated with a significantly lower ulcer relapse rate.

Elevated blood flow at the margins of ulcers has been shown to be important for healing to occur (28). Given that H_2S is a vasodilator, it is possible that this may be one of the mechanisms through which H_2S promotes healing. Fiorucci *et al.* (8) noted that H_2S donors enhanced blood flow in the stomach. Other endogenous substances that have been shown to promote ulcer healing, such as prostaglandin E_2 and nitric oxide, are believed to do so in part by increasing blood flow at the ulcer margin (28). It is thus noteworthy that an H_2S donor was able to reverse

the detrimental effects of diclofenac, an inhibitor of prostaglandin synthesis, on ulcer healing. The potential contributions of the vascular actions of H₂S to its beneficial effects in ulcer healing require further investigation.

As mentioned above, prostaglandins are known to make important contributions to mucosal defense and to ulcer healing (28). It was possible that H₂S donors might enhance healing of gastric ulcers by further increasing gastric prostaglandin synthesis. In the present study, we observed a very large increase in gastric PGE₂ synthesis in the tissue from rats with ulcers *vs.* controls. Similarly, and not surprising given the substantial infiltration of neutrophils in the ulcer bed, there was also a substantial increase in LTB₄ production by gastric tissue from rats with ulcers *vs.* controls. Treatment with either of two H₂S donors (Lawesson's reagent and 4HTB) did not affect the rates of synthesis of these two eicosanoids.

H₂S has been known for many years to be able to modulate metabolic functions in various cells. Recently, inhaled H₂S was shown to produce a hibernation-like state in mice (29). H₂S has also recently been shown to be a substrate for mitochondrial respiration (30). It is possible, therefore, that the beneficial effects of H₂S on healing observed in the present study may have been in part related to modulatory effects of this gaseous mediator on metabolism.

In summary, this study provides evidence that H₂S is an endogenous regulator of wound healing, and such healing can be markedly enhanced through administration of H₂S donors. Thus, enhancement of endogenous H₂S synthesis or delivery of appropriate concentrations of H₂S may have clinical utility in enhancing the healing of wounds, including gastrointestinal ulcers. **FJ**

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