

BASIC-ALIMENTARY TRACT

Inhibition of Hydrogen Sulfide Generation Contributes to Gastric Injury Caused by Anti-Inflammatory Nonsteroidal Drugs

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Background & Aims: Hydrogen sulfide (H₂S), an endogenous gaseous mediator that causes vasodilation, is generated in mammalian tissues by cystathionine β-synthase (CBS) and cystathionine-γ-lyase (CSE). Here, we have investigated the role of H₂S in a rodent model of nonsteroidal anti-inflammatory drug (NSAID) gastropathy. **Methods:** Rats were given acetyl salicylic acid (ASA) or an NSAID alone or in combination with NaHS, an H₂S donor, and killed 3 hours later. Gastric blood flow was measured by laser-Doppler flowmetry, whereas intravital microscopy was used to quantify adhesion of leukocytes to mesenteric postcapillary endothelium. **Results:** At a dose of 100 μmol/kg, NaHS attenuated by 60%–70% the gastric mucosal injury, and tumor necrosis factor (TNF)-α, intercellular adhesion molecule (ICAM)-1, and lymphocyte function-associated antigen (LFA)-1 mRNA up-regulation induced by NSAIDs (*P* < .05). NaHS administration prevented the associated reduction of gastric mucosal blood flow (*P* < .05) and reduced ASA-induced leukocyte adherence in mesenteric venules. NaHS did not affect suppression of prostaglandin E₂ (PGE₂) synthesis by NSAIDs. Glibenclamide, a K_{ATP} channel inhibitor, and DL-propargylglycine, a CSE inhibitor, exacerbated, whereas pinacidil, a K_{ATP} opener, attenuated gastric injury caused by ASA. Exposure to NSAIDs reduced H₂S formation and CSE expression (mRNA and protein) and activity by 60%–70%. By promoter deletion and mutation analysis, an Sp1 consensus site was identified in the CSE promoter. Exposure to NSAIDs inhibits Sp1 binding to its promoter and abrogates CSE expression in HEK-293 cells transfected with a vector containing the core CSE promoter. Exposure to NSAIDs inhibits Sp1 and ERK phosphorylation. **Conclusions:** These data establish a physiologic role for H₂S in regulating the gastric microcirculation and identify CSE as a novel target for ASA/NSAIDs.

Gaseous transmitters are a growing family of regulatory molecules involved in multilevel regulation of physiologic and pathologic functions in mammalian tissues.¹ Although nitric oxide (NO) is the best characterized member of this family, it is increasingly recognized that carbon monoxide (CO) and hydrogen sulfide (H₂S) might also function as gaseous mediators in mammalian cells.^{1,2}

H₂S synthesis from cysteine occurs naturally in a range of mammalian tissues principally through the activity of 2 pyridoxal-5'-phosphate-dependent enzymes, cystathionine γ lyase (CSE) and cystathionine β synthetase (CBS), although alternative sources (eg, by activity of cysteine aminotransferase and/or 3-mercaptosulphurtransferase) cannot yet be discounted.¹⁻⁴ In some tissues, CBS and CSE are both required for generation of H₂S, whereas, in others, one enzyme suffices. CBS-derived H₂S is a physiologically relevant neurotransmitter in the central nervous system in which exposure to this gaseous transmitter¹ results in activation of adenosine triphosphate (ATP)-sensitive K⁺ (K_{ATP}) channels. In the cardiovascular system, H₂S, mostly derived from CSE, modulates endothelium-dependent and -independent vasodilation.^{1,5,6} Highlighting the functional role of H₂S, CBS deficiency leads to hyper-homocyst(e)inemia, a condition that includes elevated serum levels of homocysteine, homocystine, or homocysteine-mixed disulfides, and is associated with increased blood pressure and endothelial dysfunction.⁷ In rodents, CBS/CSE deficiency induced by

Abbreviations used in this paper: CBS, cystathionine β-synthase; CSE, cystathionine-γ-lyase; H₂S, hydrogen sulfide; MPO, myeloperoxidase; NO, nitric oxide; PGE₂, prostaglandin E₂.

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genetic-deletion^{8,9} or chronic treatment with DL-propargylglycine, an irreversible and selective inhibitor of CSE,¹⁰ results in reduced nitric oxide (NO) bioactivity. This leads to severe endothelial dysfunction characterized by impaired aortic relaxation to acetylcholine and a paradoxical vasoconstriction of mesenteric microvessels in response to bradykinin.⁹

Gastrointestinal injury is a common complication of nonsteroidal anti-inflammatory drugs (NSAIDs) and acetylsalicylic acid (ASA) therapy.¹¹ Owing to the inhibition of cyclooxygenase (COX) isoenzymes in the gastrointestinal tract, ASA and NSAIDs reduce the intrinsic ability of the gastric mucosa to resist injury induced by endogenous and exogenous agents.^{11,12} Thus, inhibition of generation of COX-1- and COX-2-derived eicosanoids (prostaglandin E₂ [PGE₂] and the lipoxin analogue, aspirin-triggered lipoxin) results in altered gastric mucosal blood flow and increased leukocyte-endothelial adhesive interactions in the gastric microcirculation, an essential step in the process of acute gastric injury caused by ASA/NSAIDs.^{13–15} Human and animal studies have highlighted the role of gaseous mediators, particularly NO, in maintaining gastric mucosal integrity.^{16,17} Thus, by modulating expression/activity of adhesion molecules at the leukocyte-endothelium interface and by maintaining gastric mucosal blood flow, NO compensates for depressed generation of protective eicosanoids.¹⁸ Whether or not the gastric mucosa has the ability to generate H₂S and the regulatory functions exerted by this gaseous mediator in this tissue is unknown.

Materials and Methods

Materials

Aspirin; indomethacin; ketoprofen; diclofenac; lipopolysaccharide (LPS; *Escherichia coli* 0111:B4 serotype); sodium hydrogen sulfide (NaHS); L-cysteine, glibenclamide, a K_{ATP} channel blocker; pinacidil, a K_{ATP} opener; DL-propargylglycine; antiphosphoserine antibody; and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The stock solution of NaHS was freshly prepared by dissolving NaHS immediately before use. All tissue culture reagents, including minimal essential medium (MEM), fetal bovine serum (FBS), penicillin, and streptomycin, were obtained from Gibco (Milan, Italy). Filtered, deionized water was used for buffer preparation. Silver and sulphide ion selective electrodes were from ThermoOrion (Beverly, MA). The anti-human Sp1 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-ERK and anti-phospho-ERK antibody were obtained from New England Biolabs (Beverly, MA). The anti-CSE antibody was a generous gift of Dr. N. Nishi, Department of Endocrinology, Kagawa Medical School, Kagawa, Japan.

Acute Damage

All studies were approved by the animal study committees of the University of Perugia and the University of Calgary. Male Wistar rats (225–275 g) were obtained from Charles River Breeding Farms (Monza, Italy, or Montreal, Canada) and maintained on standard laboratory rat chow on a 12-hour light/dark cycle. Rats were deprived of food for 24 hours before being given one of the following orally: ASA (30 mg/kg), indomethacin (10 mg/kg), ketoprofen (30 mg/kg), ASA (30 mg/kg) plus celecoxib (100 mg/kg), or vehicle.^{13–15} Rats were killed 3 hours later, and gastric mucosal damage was measured. To assess gastric mucosal damage, the lengths (in mm) of all lesions were measured with a digital calliper, and a “gastric damage score” was calculated for each stomach by summing these values.¹³ This assessment was performed by an individual blinded to the treatments the rats had received. In addition, samples of the body region of the stomach were excised and processed, as described previously,¹⁵ for measurement of myeloperoxidase (MPO) activity as an index of leukocyte accumulation in the tissue.

To investigate whether H₂S administration could prevent the gastric injury caused by NSAIDs, rats were treated intraperitoneally with NaHS, as the H₂S donor, at doses of 25, 50, 100, or 150 μmol/kg or with vehicle, and 30 minutes later were given ASA orally at a dose of 50 mg/kg.^{13–15} The rats were killed 3 hours later, and gastric mucosal damage and MPO activity were evaluated as described above. In these experiments, NaHS was used as the H₂S donor for the following reasons: (1) NaHS dissociates to Na⁺ and HS⁻ in solution, then HS⁻ associates with H⁺ and produces H₂S. At physiologic pH, ≈one third of the H₂S exists as the undissociated form (H₂S), whereas the remaining two thirds exists as HS⁻ at equilibrium with H₂S; (2) the use of NaHS enables us to define the concentrations of H₂S in solution more accurately and reproducibly than bubbling H₂S gas; (3) the influence of Na⁺ ions (less than 1 mmol/L) is negligible; and (4) NaHS at concentrations used in the present study does not change the pH of the medium.

To investigate the role of endogenous H₂S in modulating gastric mucosal resistance to damage induced by ASA and NSAIDs, rats were pretreated with DL-propargylglycine^{5,10} at a dose of 10 mg/kg per day for 5 days and then administered ASA (50 mg/kg). The rats were killed 3 hours later, and gastric mucosal injury and MPO were assessed. In another set of experiments, we determined whether or not cysteine or N-acetylcysteine, which are known to release H₂S after enzymatic degradation by CSE and CBS, would influence gastric resistance to ASA-induced damage. Rats were treated with cysteine (15 mg/kg) or N-acetylcysteine (15 mg/kg) prior to oral administration of ASA (50 mg/kg). Three hours later, the extent of gastric damage was assessed, as described previously.

Finally, to determine whether K_{ATP} channels are involved in the gastric protection afforded by H₂S,⁶ rats were pretreated intraperitoneally (IP) with NaHS (100 μmol/kg) alone or in

combination with glibenclamide (10 mg/kg), a K_{ATP} channel blocker, or pinacidil, a K_{ATP} channel opener, immediately prior to oral administration of ASA (50 mg/kg). Three hours later, the extent of mucosal injury and tissue MPO activity were assessed as described previously.

Gastric Mucosal Blood Flow

Rats were anesthetized with ketamine and pentobarbital sodium (50 mg/kg, IP), and body temperature was maintained at 37°C by means of a thermostatically regulated heating pad. Gastric blood flow was measured using a laser-Doppler flowmeter (Perimed, Stockholm, Sweden).¹⁹ The stomach was exposed by a midline laparotomy, opened by an incision along the greater curvature, and then pinned over a plexiglas platform and clamped. A "mucosal chamber" of 1.5 cm² gastric mucosa was prepared.¹⁹ Throughout the experiment, the mucosa was bathed with 0.1 mmol/L HCl, maintained at 37°C by means of circulating warm water. Blood flow was measured after 30 minutes of equilibration and recorded continuously throughout the experiments from the mucosal side of the stomach with a laser probe placed 0.5–1 mm above the mucosal surface in the acidified solution. H₂S (10 mg/kg) was administered IP at the end of the equilibration period. ASA (30 mg/kg) was administered orally 1 hour before the start of the experiment. The left carotid artery was cannulated and connected to a pressure transducer for mean arterial pressure (MAP) recording using a multichannel recorder (PowerLab PC; A.D. Instruments, Milford, MA). Four rats per group were used for these studies, and measurements were repeated at least 3 times.

Intravital Microscopy

Rats were anesthetized with pentobarbital sodium (60 mg/kg, IP), and cautery incisions were made along the abdominal region. A tracheotomy was performed to facilitate breathing. The rats were placed in a supine position, and a segment of the mesentery was exteriorized through the abdominal incision. The mesentery was carefully placed over an optically clear viewing pedestal that allowed for transillumination of a 2-cm² segment of tissue. All exposed tissue was covered with saline-soaked gauze to minimize dehydration. The temperature of the pedestal was kept at 37°C, and the mesentery was superfused with warmed bicarbonate-buffered saline (pH 7.4). An intravital microscope (Nikon L25/0.35) and a ×10 eyepiece were used to observe the mesenteric microcirculation. Postcapillary venules with diameters ranging from 20 to 40 μm were selected for the study. A video camera mounted on the microscope (Panasonic digital 5000) projected the image onto a monitor, and the images were recorded for playback analysis using a videocassette recorder. Images of the mesenteric microcirculation were recorded for 15 minutes before aspirin administration (baseline), at the time of aspirin administration (time 0), and every 15 minutes for 60 minutes. Aspirin was administered intragastrically at a dose of 50 mg/kg. In some experiments, rats were pretreated intragastrically with NaHS (100 μmol/kg) 30 minutes prior to ASA admin-

istration (controls received vehicle at the same time). In other experiments, glibenclamide (10 mg/kg) was administered 15 minutes prior to NaHS administration. Leukocyte adhesion was blindly quantified as the number of leukocytes that adhered to the vessel wall for 30 seconds or more along a 100-μm venule length.¹³

Analysis of H₂S Production by Gastric Mucosa

Sulfide concentrations and production by the gastric mucosa were measured as previously described^{20,21} with minor modifications. Briefly, gastric samples (100–150 mg) were homogenized in 1 mL ice-cold T-PER protein extractor. A tissue sample containing ~5–10 mg protein was also lysed in 1 mL ice-cold T-PER protein extractor. Two milliliters of an assay reaction mixture was introduced into the reactor. The mixture contained 10 mmol/L L-cysteine (or N-acetyl-L-cysteine), 2 mmol/L pyridoxal 5'-phosphate, 100 mmol/L potassium phosphate buffer (pH, 7.4), and 10% (wt/vol) liver homogenate or gastric tissue lysate. DL-propargylglycine, 2 mmol/L, was incubated with gastric homogenates for 5 minutes at 37°C prior to the enzyme reaction. Calmodulin and calcium chloride were added to a final reaction mixture at concentrations of 9.6 μmol/L and 0.6 mmol/L, respectively. A constant stream of nitrogen was passed through the mixture via gas-inlet capillary. Reactions were initiated by transferring the reactor from ice bath to a 37°C water bath. The stream of nitrogen carried the sulfide acid in the second reactor containing 2 mL sulfide antioxidant buffer (SAOB) solution, consisting of 2 mol/L KOH, 1 mol/L salicylic acid, and 0.22 mol/L ascorbic acid at pH 12.8. After incubating at 37°C for 90 minutes, 1 mL 50% trichloroacetic acid solution was added to the mixture to stop the reaction. The remainder of H₂S in the mixture was carried out via nitrogen stream by another 30 minutes of incubation at 37°C. The concentration of sulfide in the SAOB solution was measured with a sulfide-sensitive electrode Model 9616 S²⁻/Ag⁺ electrode (Orion Research, Beverly, MA).

Gastric Expression of CSE and CBS

Total RNA was isolated from rat liver and from gastric tissue by using the TRIzol reagent according to manufacturer specifications (Invitrogen, Milan, Italy). RNA was processed directly to cDNA by reverse transcription with Superscript III (Invitrogen). Briefly, 2 μg RNA was added to the mixture, which contained DNase I reaction buffer 10X and 1 U DNase I. The mixture was incubated for 15 minutes at room temperature, then 1 μL EDTA 25 mmol/L solution was added, and the mixture was incubated at 95°C for 5 minutes. Four microliters of first-strand buffer 5X (250 mmol/L Tris-HCl, pH, 8.3; 375 mmol/L KCl; 15 mmol/L MgCl₂) were mixed with 2 μL of DTT 0.1 mol/L, 1 μL of dNTPs mix 10 mmol/L, 0.5 μL of random primers 300 ng/μL, 1 μL of RNase out, and 1 μL of Super Script III. The mixture was incubated at room temperature for 10 minutes and at 42°C for 50 minutes, heated at

95°C for 5 minutes to inactivate the enzyme, and cooled at 4°C. All PCR primers for quantitative and qualitative PCR were designed using software PRIMER3-NEW using published sequence data from the NCBI database. Primers were synthesized by MWG BIOTECH. For rat GAPDH, the sense primer was 5'-ATGACTCTACCCACGGCAAG-3', and the antisense was 5'-TACTCAGCACCAGCATCACC-3'; for rat CBS, the sense primer was CCAGGACTTGGAGGTACAGC, and the antisense was TCGGCACTGTGTGGTAATGT; for rat CSE, the sense primer was GTATTGAGGCACCAACAGGT, and the antisense was GTTGGGTTTGTGGGTGTTTC; for COX-1, the sense primer was 5'-CGAGGATGTCATCAAGGAG-3', and the antisense was 5'-TCAGTGAGGCTGTGTTAACG-3'; for COX-2, the sense primer was 5'-TCAAGACAGATCAGAAGCGA-3', and the antisense was 5'-TACCTGAGTGTCTTTGATTG-3'; for rat TNF- α , the sense primer was CCC CAT TAC TCT GAC CCC TT, and the antisense was AGG CCT GAG ACA TCT TCA GC; for ICAM-1, the sense primer was CAAACGGGAGATGAATGG, and the antisense was TGGCGGTAATAGGTGTAAAT. In control experiments with 3 replicates, no false positives were detected. Amplification reactions contained 2 μ L cDNA, 12.5 μ L of the 2X dynamo SYBR Green Qpcr Master Mix, and 0.75 μ L of each of the specific primers. Primer concentrations in the final volume of 25 μ L were 300 nmol/L. All reactions were performed in triplicate in an iCycler iQ system (Bio-Rad, Hercules, CA), and the thermal cycling conditions were as follows: 15 minutes at 95°C, followed by 40 cycles of 95°C for 10 seconds, 55°C for 10 seconds, and 72°C for 20 seconds.

Western Blot Analysis of CSE

Tissues were quickly removed from rats and homogenized with a Teflon tissue grinder in ice-cold buffer (100 mmol/L sodium phosphate, [pH 7.8]/1 mmol/L PMSF), and sonicated. The homogenates were centrifuged at 10,900g for 5 minutes at 4°C, and the supernatants were further centrifuged at 17,400g for 20 minutes at 4°C. The resulting supernatants were quickly frozen in liquid nitrogen and stored at -80°C until use. Tissue samples were solubilized in the SDS-sample buffer, boiled for 5 minutes, separated on a 10% SDS/polyacrylamide gel, and transferred to the nitrocellulose (0.45 mm, Millipore). The CSE protein was detected with anti-CSE antibody²² at 1:3000 dilution, followed by incubation with horseradish-peroxidase-conjugated anti-rabbit IgG secondary antibody and the ECL Western blotting system (Amersham Biosciences).

Measurement of CSE Activity

CSE activity was assessed by a sensitive method recently reported by Ogasawara et al,²³ with minor modifications. Briefly, CSE catalyses the pyruvate formation from b-chloro-L-alanine. This reaction is terminated by the addition of DL-propargylglycine. The produced pyruvate is oxidized by pyruvate oxidase in the presence of thiamine pyrophosphate and bivalent magnesium to liberate H₂O₂.

A leuco dye, *N*-(carboxymethylamino)-4,4'-bis(dimethylamino)-diphenylamine, is oxidized by H₂O₂ with peroxidase to produce Bindschedler's green. The reaction was performed in 96-well dishes, and the absorbance of green dye (727 nm) was measured by a microplate reader.

Cloning of Mouse CSE Promoter Region and Construction of Reporter Plasmids

The 182-bp genomic DNA fragment upstream from the transcriptional start site (-155 to +27), containing canonical TATA and CAAT boxes and the Sp1 site, was isolated by PCR from mouse hepatocyte genomic DNA.²⁴ Briefly, the cDNA was amplified using 100 ng genomic DNA as template and utilizing *Phusion* DNA polymerase (FINNzymes, Espoo, Finland) in a 50 μ L volume reaction containing 1X *Phusion* reaction buffer, 200 nmol/L dNTPs, and 300 nmol/L each primer. The sequences of the primers used were as follows: CSE-Prom-Fwd (-155/-134), 5'-CACTGCTCTGTGCCACTGGGAG-3'; CSE-Prom-Rev (+4/+27), 5'-GTAGGAGTGCGAGGTGTTGCTTTG-3'. The 182-bp fragment was cloned into pCR-BluntII-TOPO (Invitrogen), according to the manufacturer's instructions, and sequenced. For luciferase assays, the reporter plasmid pGL3 (-155/+27)-CSE-Prom-Luc was constructed by subcloning CSE-Promotor sequence (-155 to +27) from pCR-BluntII-TOPO into the *KpnI* and *XbaI* sites of pGL3-Enhancer vector (Promega). Briefly, the pCR-BluntII-TOPO (-155/+27)-CSE-Prom construct was digested with *KpnI* and *XbaI*, and the resulting promoter digestion products were gel purified and cloned into a pGL3 enhancer vector (Promega), digested with *KpnI* and *XbaI*, creating the reporter vector pGL3 (-155/+27)-CSE-Promoter. The SP1 response element (5'-GAGGCGGGC-3') in the 182-bp fragment was mutated (5'-GATTCGTTGC-3') into pCR-BluntII-TOPO (Invitrogen) by using QuickChange Site-Directed Mutagenesis Kit (Stratagene), and oligonucleotide GCCACTGGGATTCGGGGCAGGAACGATC, and its complementary oligonucleotide according to the manufacturer's recommendations. The mutated SP1 response element is in capital letters, and the mutations are underlined and in italic letters. The fragment (-155/+27)-CSE-Prom with SP1 binding site mutated was excised from pCR-BluntII-TOPO with *KpnI* and *XbaI* enzymes and subcloned into *KpnI* and *XbaI* sites of pGL3-Enhancer vector (Promega) to generate vector pGL3 (-155/+27)Mutant-CSE-Prom. All constructs were verified by restriction site digestion and sequence analysis.

Cell Culture, Transfection, and Luciferase Assay

For luciferase assay, HEK-293 cells were cultured in DMEM supplemented with 1% penicillin/streptomycin, 1% L-glutamine, and 10% fetal bovine serum (high glucose) (Invitrogen). Cells were grown at 37°C in 5% CO₂. All the transfections were made using a calcium phosphate coprecipitation method in the presence of 25 μ mol/L chloroquine as inhibitor for DNA degradation. Transient transfections were performed using 500 ng reporter vector pGL3 (-155/+27)-

CSE-Prom, 200 ng pCMV- β -gal as internal control for transfection efficiency. The pGEM vector was added to normalize the amounts of DNA transfected in each assay (2.5 μ g). Thirty-six to 48 hours posttransfection, the cells were stimulated with ASA, indomethacin, ketoprofen, and diclofenac 50 μ mol/L for 18 hours. All agents were diluted in DMSO, and control cultures received 0.1% DMSO alone. The cells were lysed in 100 μ L diluted reporter lysis buffer (Promega), and 0.2 μ L cellular lysate was assayed for luciferase activity using Luciferase Assay System (Promega). Luminescence was measured using an automated luminometer. Luciferase activities were normalized for transfection efficiencies by dividing the relative light units by β -galactosidase activity expressed from cotransfected pCMV- β -gal plasmid. Each datum point is the average of triplicate assays. Each experiment was repeated at least 3 times.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay

Preparation of nuclear extracts from control or drug-treated cells was carried out as described previously.²⁵ The oligonucleotide 5'-CAGAGAGGGGCGGCCCGAGTG-3' was used as probe. The mutant Sp1 oligonucleotide has the sequence 5'-CAGATATCTAGATGATATCGTG-3'. The reaction mixture for EMSA contained 20 mmol/L Tris-HCl, pH 7.6, 1 mmol/L dithiothreitol, 2 mmol/L MgCl₂, 1 mmol/L EDTA, 10% glycerol, 1% Nonidet P-40, 1 μ g poly(dI-dC), and 8–10 μ g nuclear proteins. Unlabeled wild-type or mutant oligonucleotides or preimmune, Sp1, or Sp3 antibody was added into the reaction mixture and incubated for 10 minutes at room temperature. ³²P-Labeled probe DNA (300,000 cpm) was added, and the binding reaction was allowed to proceed for another 20 minutes. After reaction, mixtures were resolved on 6% polyacrylamide gels at 200 V for 2 hours. Gels were dried and subjected to autoradiography.

Gastric Prostanoid Synthesis

Gastric PGE₂ concentrations were measured according to previously published methods.^{13–15} Unless otherwise stated, 3 hours after the administration of the drugs, gastric specimens taken from ulcerated tissues were snap frozen in liquid nitrogen. Samples were then lysed in 50 mmol/L Tris-HCl (pH 8.4) buffer and finely minced. After the tissues had been washed and resuspended in 1 mL buffer, they were vortexed at room temperature for 1 minute to stimulate PGE₂ production, followed by centrifugation at 10,000g for 15 seconds at 4°C. The amounts of PGE₂ in the resulting supernatants were determined by specific ELISA (Cayman Chemical Company, Ann Arbor, MI) following manufacturer's instructions.

Statistical Analysis

All data are presented as the mean \pm SEM. Comparisons of groups of data were performed using a 1-way analysis of variance followed by the Student-Newman-Keuls post hoc

test. An associated probability (*P* value) of less than 5% was considered significant.

Results

The Gastric Mucosa Generates Physiologically Relevant Concentrations of H₂S

As illustrated in Figure 1, we found that messenger RNA (mRNA) encoding for CBS and CSE, the 2 key enzymes involved in H₂S formation from L-cysteine, were expressed in the rat gastric mucosa. Moreover, incubating gastric mucosal homogenates with L-cysteine, the natural substrate of CBS and CSE, in the presence of pyridoxal-5-phosphate, an essential cofactor for enzyme activities (see Materials and Methods section), resulted in H₂S generation (Figure 1B, *n* = 6). We found that, in the presence of L-cysteine, the gastric mucosa generated \approx 150 nmol/L/mg protein/min of gaseous H₂S and that this activity was concentration dependently inhibited by addition of DL-propargylglycine, a selective and irreversible inhibitor of CSE (Figure 1B; *n* = 6; *P* < .01 vs L-cysteine alone).

Because H₂S is a vasodilator,¹ we utilized laser Doppler flowmetry to determine whether or not it modulates gastric mucosal blood flow. As illustrated in Figure 1C, administering NaHS (100 μ mol/kg) IP to rats resulted in a transient reduction of MAP (6.0 \pm 1.6 mm Hg 10 minutes after NaHS administration; *n* = 6; *P* < .05 vs control). This effect was associated with a transient, albeit significant, increase in gastric mucosal blood flow (\approx 20%; *n* = 6; *P* < .05 vs control). Gastric vasodilation caused by NaHS was abrogated by pretreating rats with glibenclamide (10 mg/kg; *n* = 6; *P* < .05 vs H₂S). In rats given ASA (100 mg/kg), gastric mucosal blood flow was significantly reduced in comparison with control rats (Figure 1D; *n* = 7; *P* < .05 vs control). However, administration of NaHS (100 μ mol/kg) reversed the impairment of gastric mucosal blood flow caused by ASA (*n* = 6, *P* < .05 vs rats treated with ASA alone).

H₂S Protects Against Gastric Mucosal Injury Induced by ASA and NSAIDs

Administration to rats of ASA, indomethacin, ketoprofen, or diclofenac resulted in damage to the gastric mucosa (*n* = 6 rats per group, *P* < .01 vs control). The injury consisted of hemorrhagic erosions, usually linear, running along the crests of rugal folds, and localized primarily in the corpus region of the stomach. Exposure to ASA and NSAIDs resulted in a

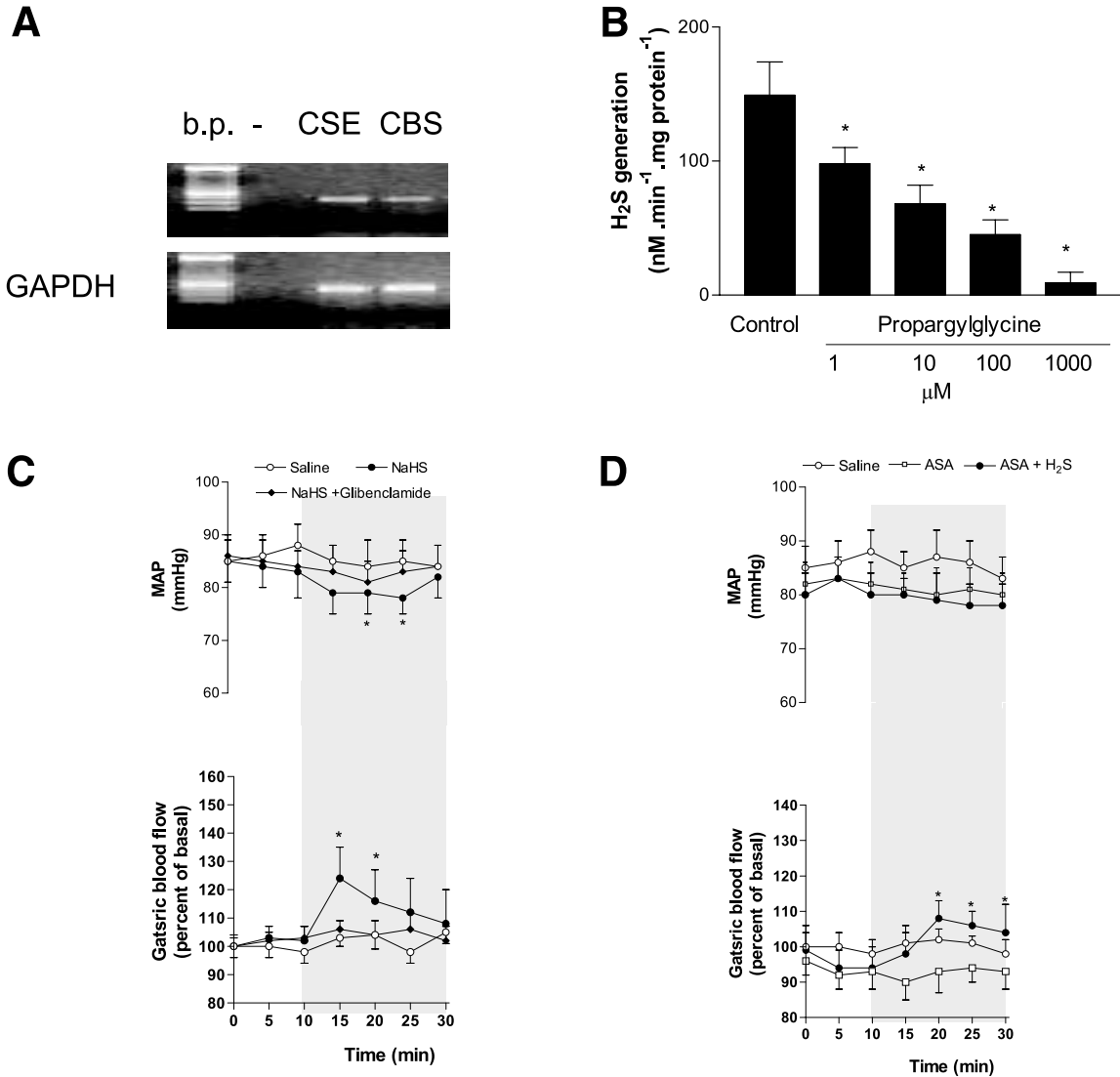


Figure 1. The rat gastric mucosa generates H₂S. (A) RT-PCR expression of CSE and CBS in the gastric mucosa. The abbreviation bp denotes base pairs. The expression of the housekeeping gene GAPDH is shown. (B) Production of H₂S by gastric mucosal homogenates. H₂S production was assessed as described in the Materials and Methods section in gastric homogenates incubated with L-cysteine and pyridoxal-5'-phosphate. Inhibition of H₂S generation by increasing concentration of DL-propargylglycine confirms the prevalent role of CSE in generation of H₂S by the stomach. Data are expressed as mean ± SEM of 6 experiments. *P* < .05 vs control. (C–D) Effect of H₂S on gastric mucosal blood flow and mean arterial pressure (MAP). Gastric blood flow was measured by laser Doppler flowmetry. NaHS, 100 μmol/kg, was administered IP 10 minutes after starting recording. Glibenclamide was administered 1 hour before starting recording. (D) Rats were treated with ASA (100 mg/kg) 1 hour before starting recording. Data are expressed as mean ± SEM of 6 rats. **P* < .05 vs baseline.

2- to 3-fold increase in MPO activity, a measure of granulocyte accumulation in the gastric mucosa, and 4- to 6-fold increase in gastric TNF-α and ICAM-1 mRNA, measures of gastric inflammation (n = 6 rats per group, *P* < .01 vs control). NSAID administration was also associated with changes in gastric mucosal PGE₂ synthesis, as well as alterations in the expression of prostanoid generating enzymes. Thus, exposure to ASA and NSAIDs reduced gastric PGE₂ synthesis by ≈90% and increased gastric expression of COX-2 mRNA by 3- to 5-fold (n = 6 rats per group, *P* < .01 vs control). In contrast, no changes in COX-1 mRNA

expression were observed. Treating rats with NaHS significantly attenuated the gastric damage caused by NSAIDs. In rats given NaHS (100 μmol/kg, IP), the gastric mucosa injury score; MPO activity; and TNF-α, ICAM-1, LFA-1, and COX-2 mRNA levels were reduced by ≈70%–80% in comparison with rats given ASA or NSAIDs alone (n = 6 rats per group, *P* < .01 vs ASA and NSAIDs). NaHS administration also attenuated histologic changes (data not shown) induced by ASA. NaHS had no effect on inhibition of gastric PGE₂ synthesis caused by ASA and NSAIDs (n = 6 rats per group, *P* > .05).

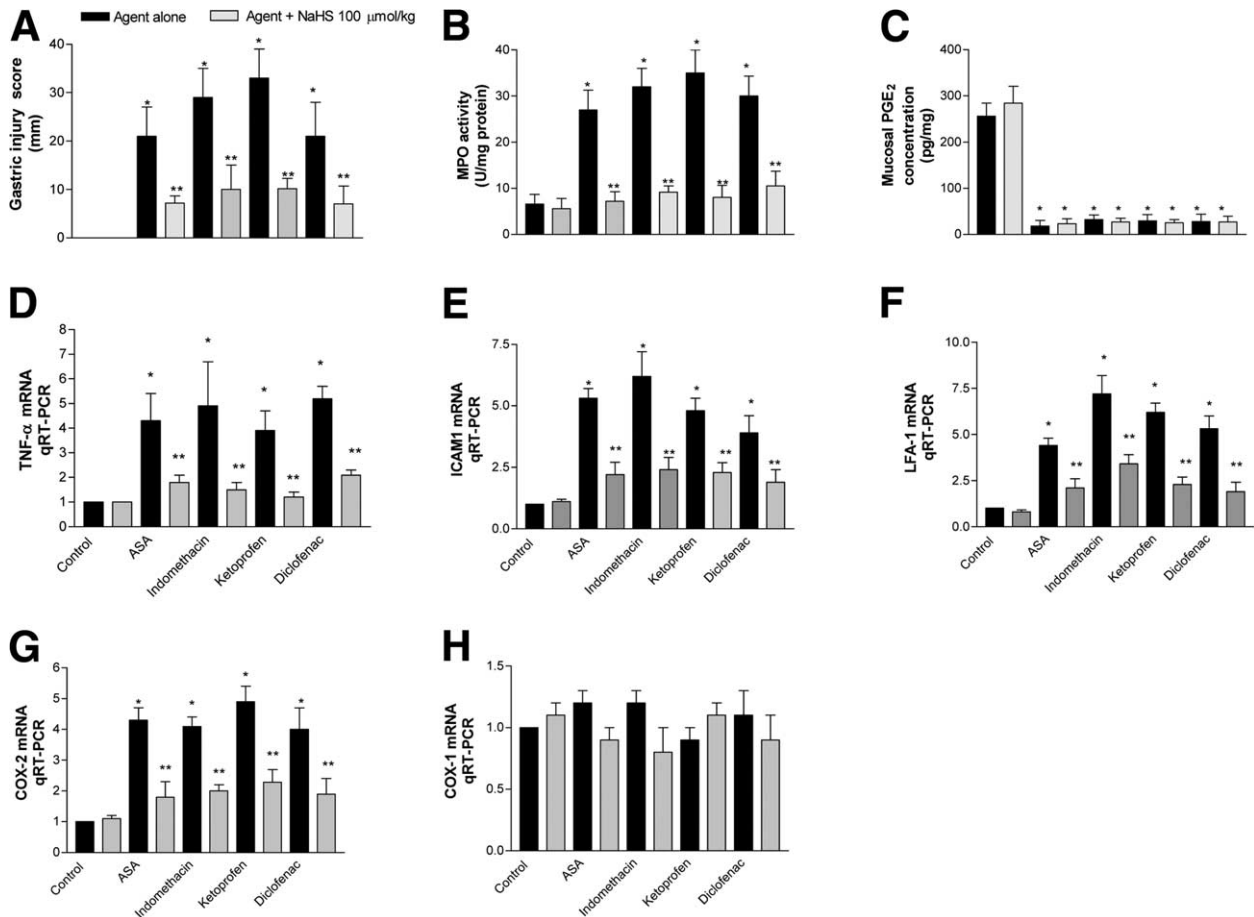


Figure 2. H₂S protects the gastric mucosa against the damage caused by ASA or NSAIDs. Rats were coadministered with ASA or NSAIDs orally and NaHS (100 μ mol/kg) intraperitoneally. (A–H) NaHS attenuates gastric injury induced by ASA (30 mg/kg), indomethacin (10 mg/kg), ketoprofen (30 mg/kg), or diclofenac (20 mg/kg). Data are expressed as mean \pm SEM of 6 rats. * P < .05 vs control rats. ** P < .05 vs rats treated with ASA or NSAIDs alone.

The protection afforded by NaHS was dose dependent: thus, although a dose of 25 μ mol/kg failed to attenuate the gastric injury caused by ASA (30 mg/kg), all of the other tested doses effectively reduced the extent of mucosal damage and MPO activity (Figure 3A and 3D; n = 6 rats per group, P < .01 vs ASA). Similar to NaHS, treating rats with L-cysteine or N-acetyl-cysteine protected against mucosal damage caused by ASA (Figure 3B, C, and F; n = 4 rats per group, P < .01 vs ASA). However, both agents failed to increase mucosal H₂S levels (Figure 3E).

Exposure to ASA and other NSAIDs resulted in a drastic reduction in the ability of gastric mucosa to generate H₂S (Figure 4; n = 6 rats per group, P < .01 vs ASA). Suppression of H₂S generation caused by these agents approached 60% (n = 6 rats per group, P < .01 vs control). By qRT-PCR and Western blot analysis, we found that AS/NSAIDs administration associates with a

significant reduction of CSE, but not CBS, expression (Figure 4B–D; n = 6; P < .01).

Direct Inhibition of CSE Expression by NSAIDs

Computer-assisted analysis of the promoter region of the mouse and human CSE has revealed the presence of a potential Sp1 consensus sequence (5'-GAGGCGGGGC-3') located within the -155/+27 region of the mouse CSE promoter. Because a previous study by Ishii et al²⁴ demonstrated that transfection of HEK-293 cells with a promoter containing this consensus site results in CSE promoter activation, we have taken advantage from this study to generate a pGL3 (-155/+27)-CSE-Promoter expression vector cloned upstream to luciferase that was used to transfect HEK-293 cells. As shown in Figure 5A, transfection with this promoter resulted in \approx 60-fold increase in luciferase expression (n = 4; P < .05 vs cells transfected with

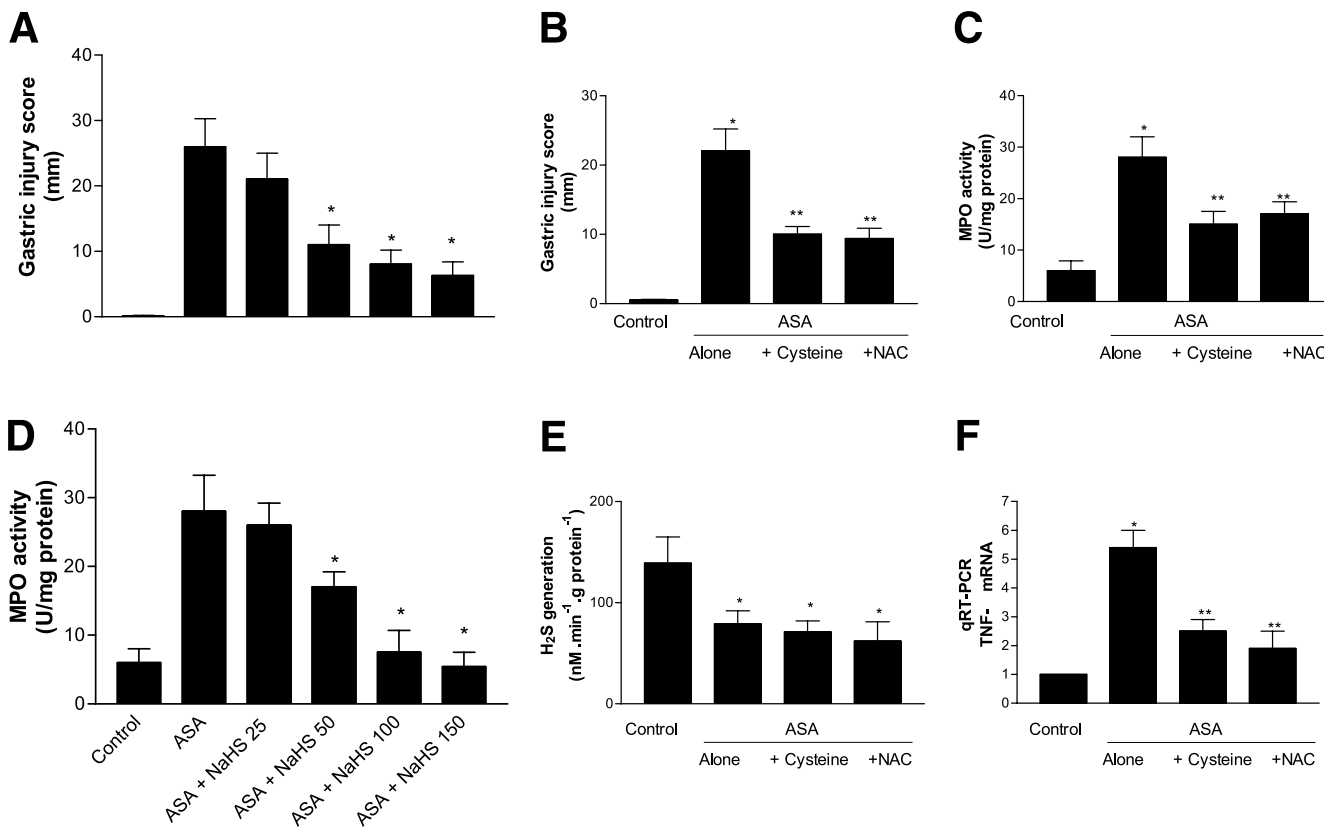


Figure 3. Gastric protection afforded by NaHS is dose dependent and is reproduced by other H₂S donating agents. (A and D) Effect of different doses of NaHS on gastric injury and gastric MPO activity induced by ASA (30 mg/kg). Data are expressed as mean ± SEM of 6 rats. **P* < .05 vs control rats. ***P* < .05 vs rats treated with ASA alone. (B, C, E, and F) L-cysteine and N-acetylcysteine (15 mg/kg) protect against mucosal injury and changes in MPO activity, H₂S generation, and TNF-α mRNA expression caused by ASA (30 mg/kg). Data are expressed as mean ± SEM of 6 rats. **P* < .05 vs control rats. ***P* < .05 vs rats treated with ASA alone.

the empty vector). Treating cells with indomethacin almost completely abrogated this effect (*n* = 4; *P* < .05 vs cells transfected with pGL3). Similar to indomethacin, ketoprofen and ASA (0.1, 0.1, and 3 mmol/L, respectively) reduced CSE transactivation in this experimental setting (not shown). To investigate whether the Sp1 binding site was involved in CSE regulation,²⁶ we transfected HEK-293 cells with a vector carrying on a mutated Sp1 binding site (Figure 5B and C), and, as shown in Figure 5A, we found that the mutation of the Sp1 consensus site not only reduced the expression of CSE by ≈6 fold in comparison with the cells transfected with the intact Sp1 binding site but greatly attenuated inhibition caused by indomethacin (*n* = 4; *P* > .05), indicating that this site regulates either CSE promoter activity as well as sensitivity of CSE to indomethacin. To determine whether inhibition of CSE promoter activity by indomethacin is caused by changes in the interaction between nuclear proteins with the promoter, an EMSA was performed with oligonucleotides corresponding to the region (−135 to −125) of the wild-type promoter of CSE. As shown in Figure 5C, we found that NSAID treatment (Figure 5C, lanes 4–7) significantly reduced the binding of

nuclear proteins to the probes. Our data also show that interaction of nuclear proteins with the Sp-1 probe is sequence specific because the binding could be competed away by an excess of unlabeled wild-type oligonucleotide (Figure 5C, lane 10) but not by mutant oligonucleotide (Figure 5C, lane 9). We next tested whether the nuclear proteins that bind to this region are Sp1-related proteins. As shown in Figure 5C, lane 8, the addition of a Sp1 antibody abrogated the signal. These data suggest that Sp1 is constitutively bound to the Sp1 site located between bp 135 and 125 in the CSE promoter and that this binding is suppressed by NSAIDs. Because Sp1 is a phosphoprotein,^{27,28} we have investigated whether NSAIDs might modulate its phosphorylation status. Control or drug-treated cells were harvested, and Sp1 was immunoprecipitated by an anti-Sp1 antibody. The immunoprecipitates were subjected to SDS-PAGE and probed with antiphosphoserine antibody. As shown in Figure 5D, phosphorylation of Sp1 (*n* = 3; Figure 5D, lower) but not the total amount of the protein (Figure 5D, upper) was significantly reduced by incubation with indomethacin, 100 μmol/L (*n* = 4).

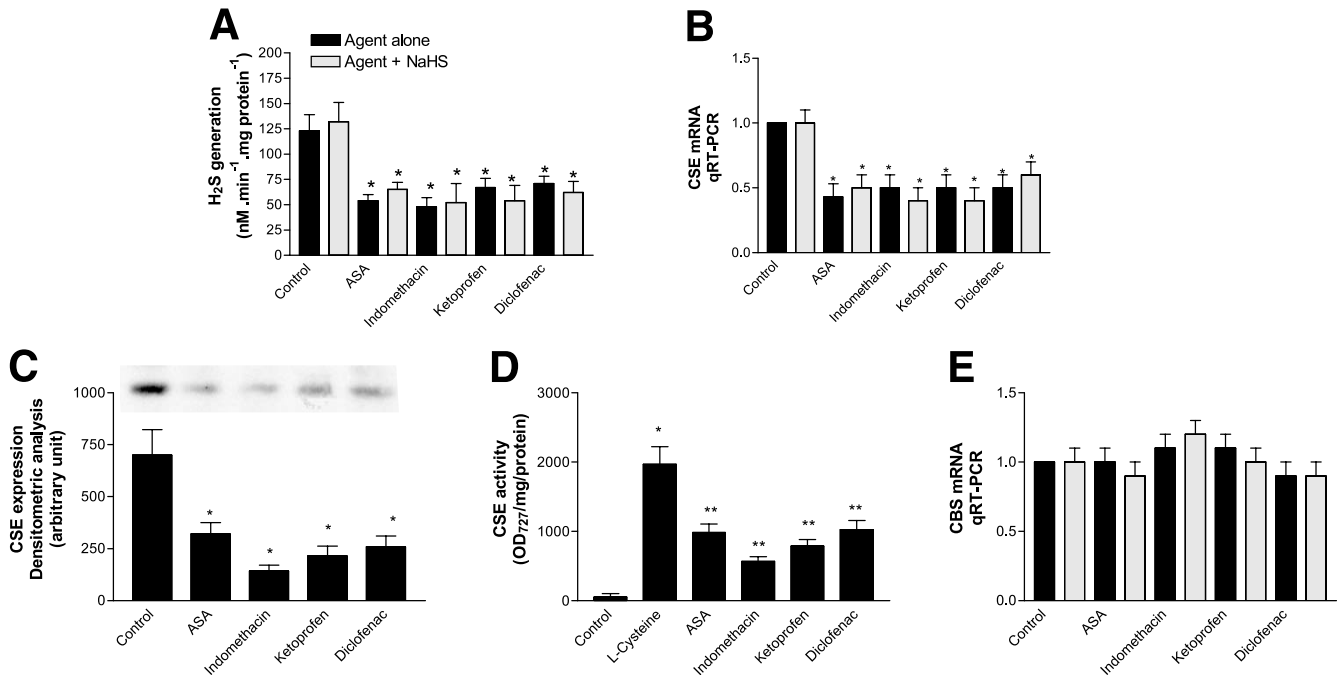


Figure 4. Exposure to ASA or NSAIDs reduces H₂S generation and CSE expression/activity in the gastric mucosa. (A) H₂S generation is significantly impaired in rats exposed to ASA or to NSAIDs. Data are expressed as mean \pm SEM of 6 rats. * P < .05 vs control rats. (B–D) ASA and NSAIDs reduce CSE activity as measured by (B) qRT-PCR, (C) Western blot analysis, and (D) activity measurements. (C) The Western blot analysis and relative densitometric analysis. Data are expressed as mean \pm SEM of 6 rats. * P < .05 vs control rats. E qRT-PCR analysis of CBS expression in the gastric mucosa. Data are expressed as mean \pm SEM of 6 rats. * P < .05 vs control rats.

Because one of the protein kinases that phosphorylates Sp1 is ERK2,^{27,29,30} we tested whether NSAIDs reduce ERK phosphorylation. As shown in Figure 5E, indomethacin inhibited basal ERK activity ($n = 3$). Exposure of HEK-293 cells to PD98059, a specific ERK inhibitor, reduced the Sp1 phosphorylation and reduced CSE expression ($n = 3$; data not shown).

CSE Inhibition Exacerbates Gastric Injury Caused by NSAIDs

Confirming the regulatory role of H₂S on maintaining gastric mucosal integrity, inhibition of CSE activity with DL-propargylglycine (10 mg/kg) caused a drastic reduction of H₂S generation and exacerbated the acute mucosal injury caused by oral administration of ASA (Figure 6; $n = 6$, $P < .05$ vs ASA). Furthermore, DL-propargylglycine administration caused a slight, although significant increase in ICAM-1 and LFA-1 expression in the gastric mucosa (Figure 6; $n = 6$, $P < .05$ vs control).

Protection Exerted by H₂S Involves K_{ATP} Channels

To investigate the possibility K_{ATP} channels were involved in the protection afforded by H₂S, we treated rats with glibenclamide (10 mg/kg), a K_{ATP} channel blocker, and pinacidil (10 mg/kg), a K_{ATP} opener. As shown in

Figure 7, glibenclamide reversed the protection afforded by NaHS (100 μ mol/kg) against the acute gastric damage caused by ASA; increased MPO activity; and reversed the attenuation of COX-2, TNF- α , ICAM-1, and LFA-1 mRNA expression caused by NaHS administration to ASA-treated rats (Figure 7; $n = 6$, $P < .01$ vs ASA plus H₂S). In contrast, administering rats with pinacidil significantly attenuated gastric mucosal injury caused by ASA (Figure 7A–I).

Because glibenclamide reversed the down-regulation of adhesion molecules caused by H₂S, we determined whether H₂S exerts antiadhesive effects in vivo and whether these effects were reversed by glibenclamide. These experiments were performed using intravital microscopy. In rats treated only with vehicle, an average of 1 or 2 leukocytes (per 100- μ m vessel length) were adherent to the endothelium of mesenteric venules under basal conditions, and this did not change significantly over the course of the experiment. Intra-gastric administration of ASA resulted in a progressive increase in the number of leukocytes adhering to the vessel wall, as shown in Figure 8. Pretreatment with NaHS (100 μ mol/kg) completely inhibited leukocyte adherence induced by ASA ($n = 5$, $P < .05$ vs ASA). However, when glibenclamide was administered prior to NaHS, the inhibition of

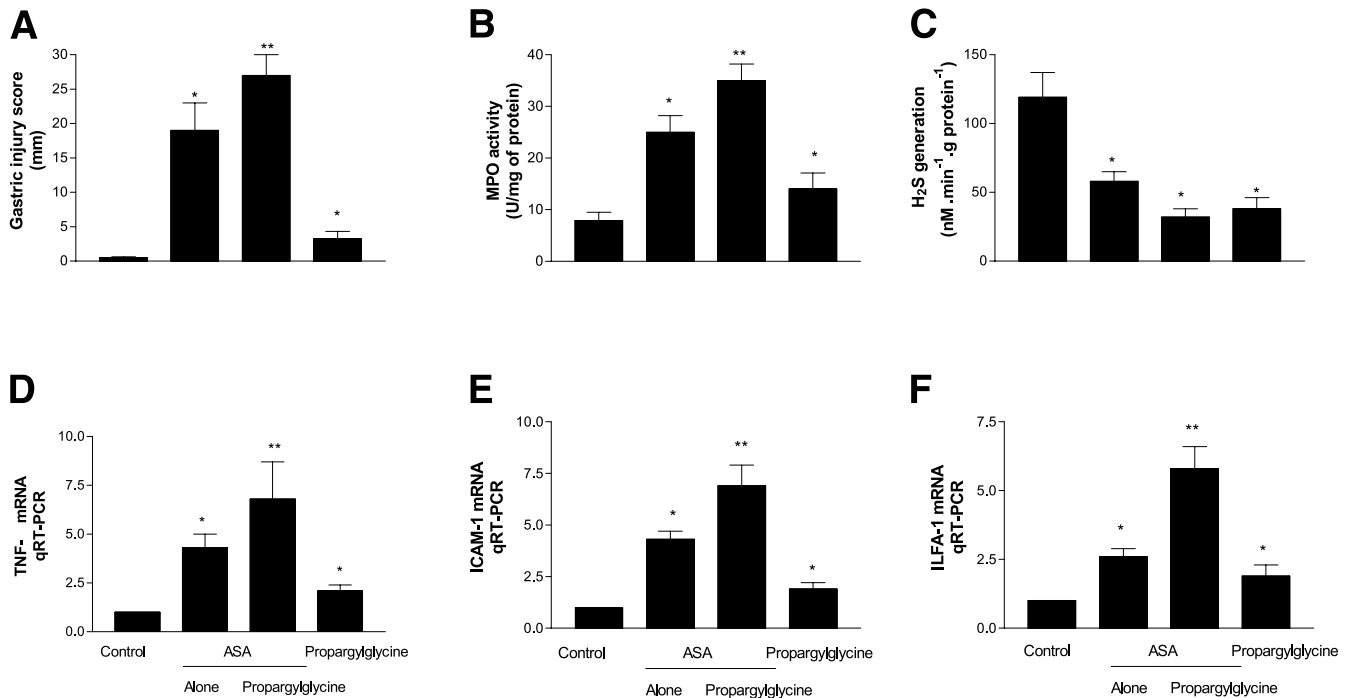


Figure 6. Effect of inhibition of CSE activity by DL-propargylglycine on gastric injury caused by ASA (30 mg/kg). Administration of DL-propargylglycine to rats exacerbates the mucosal injury caused by ASA (A), increases MPO activity (B), abrogates H₂S production by the gastric mucosa (C), and increases TNF- α , ICAM-1, and LFA-1 mRNA as measured by qRT-PCR (D–F). Data are expressed as mean \pm SEM of 6 rats. * P < .05 vs control rats.

mammalian tissues. H₂S is mainly generated by the enzymatic breakdown of L-cysteine by 2 pyridoxal-5'-phosphate-dependent enzymes: CBS and CSE.^{1–4} In addition, another, less important, endogenous source of H₂S¹ is the nonenzymatic reduction of elemental sulfur to H₂S using reducing equivalents obtained from the oxidation of glucose (see Figure 9). Although CBS is mainly expressed in the liver and central nervous system, CSE is found in smooth muscle cells in the cardiovascular system and in the gastrointestinal tract. Here, we have shown that, although both CBS and CSE are expressed by the gastric mucosa, CSE is the main enzyme involved in the H₂S generation in the stomach. Consistent with this concept, exposure of gastric mucosal homogenates to the DL-propargylglycine, a selective and irreversible inhibitor of CSE, inhibits H₂S formation. Generation of H₂S by the gastric mucosa depends on the presence of pyridoxal-5'-phosphate but not calmodulin, an essential cofactor for CBS,²¹ further highlighting the predominant role of this enzyme in the gastric mucosa.

The gastric mucosal blood flow, an essential factor in maintaining gastric mucosal integrity, is regulated by gaseous mediators.^{31–35} NO, the best characterized of these agents, increases mucosal blood flow, whereas inhibition of NO formation with selective and nonselective NO synthase (NOSS) inhibitors exacerbates the mucosal injury caused by NSAIDs.³³ We now provide evidence

that H₂S increases gastric mucosal blood flow. Thus, administering NaHS to rats causes a transient fall in MAP (\approx 10 mm Hg) while increasing gastric mucosal blood flow by \approx 20%.⁶ The systemic effects of H₂S are consistent with the finding that CSE is expressed in the aorta and mesenteric artery⁶ and that exposure of these vessels to physiologically relevant concentrations of H₂S (IC₅₀ = 125 μ mol/L) results in vasorelaxation.⁴ Vasodilation caused by H₂S is reversed by glibenclamide but is maintained after removal of the endothelium,⁴ suggesting that K_{ATP} channels are the targets of this effect.

Several of our results are consistent with the view that protection afforded by H₂S against the acute gastric injury caused by ASA/NSAIDs is mediated by modulation of adhesive interactions at the endothelium-leukocyte interface. First, NaHS protects against the reduction of mucosal blood flow caused by ASA. Exposure to ASA reduced mucosal blood flow by \approx 20%,³² an effect that was reversed by NaHS. Second, H₂S reduced leukocyte margination into the gastric microcirculation, as measured by MPO activity, and protected against up-regulation of TNF- α , ICAM-1, and LFA-1.^{35,36} In addition, exposure to H₂S dose dependently inhibited adherence of leukocytes to the vascular endothelium of mesenteric venules. Because NSAID-induced leukocyte adherence depends on regulation of ICAM-1 on endothelial cells and LFA-1 on leukocytes,^{35,36} these data add to the

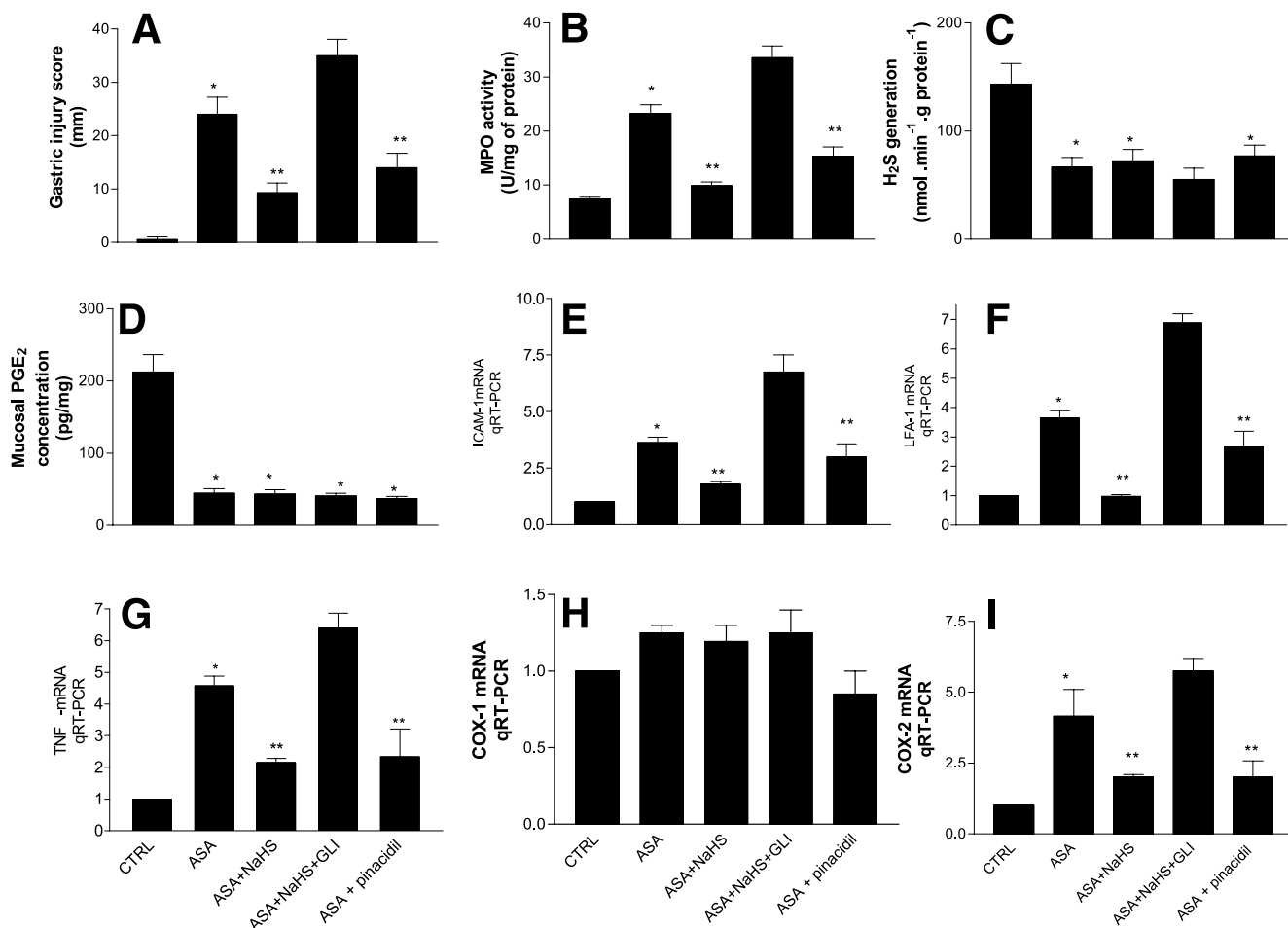


Figure 7. (A–I) Glibenclamide, a K_{ATP} channel blocker, reverses protection afforded by NaHS in rats administered ASA. Pinacidil, a K_{ATP} channel opener, reduces gastric mucosal injury caused by ASA. Rats were coadministered ASA (30 mg/kg, orally [PO]) alone or in combination with glibenclamide and pinacidil (both at the dose of 10 mg/kg, IP) and NaHS (100 μ mol/kg, IP). Data are expressed as mean \pm SEM of 6 rats. **P* < .05 vs control rats.

concept that H₂S acts locally at the leukocyte/endothelium interface. Third, the finding that anti-adhesive effects of H₂S were reversed by glibenclamide, a K_{ATP} blocker, whereas pinacidil, a K_{ATP} opener, protects against mucosal injury caused by ASA, supports the hypothesis that H₂S activates K_{ATP} channels.

An important finding of this study is the demonstration that exposure of gastric mucosa to ASA or to NSAIDs selectively decreases H₂S production by modulating CSE expression while having no effect on CBS expression. The effect on H₂S production was most profound with indomethacin, which reduced CSE activity/expression by approximately 80%, but was also observed in rats exposed to ASA, ketoprofen, and diclofenac.

The human and rodent CSE gene has recently been cloned.²⁴ The core promoter of the gene, located in the 5'-flanking region proximal to the transcriptional start site contains several putative transcriptional factor-binding sites, including MZF-1 and Sp1.²⁴ Sp1, a member of

the Sp/krüppel-like factor family,³⁷ is a ubiquitously expressed transcription factor that recognizes GC-rich sequences present in regulatory sequences of numerous housekeeping genes and is a known target for NSAIDs.²⁷ Previous studies have shown that Sp1 DNA binding activity is attenuated by NSAID treatment through a mechanism that involves inhibition of ERK phosphorylation.²⁷ Here, we demonstrated that Sp1 is a crucial transactivator for CSE gene expression because transfection of HEK-293 cells with a CSE promoter containing the Sp1 binding site stimulated CSE promoter activity,²⁴ whereas its mutation almost completely abrogated the ability of the promoter to induce CSE expression. Our results also provide evidence that NSAIDs suppress CSE expression via inhibition of the ERK/Sp1 signalling pathway. The DNA binding and transactivation activity of Sp1 is regulated by posttranslational modification (such as phosphorylation and glycosylation) (see Black et al³⁷ for review). Because Sp1 DNA binding activity, but

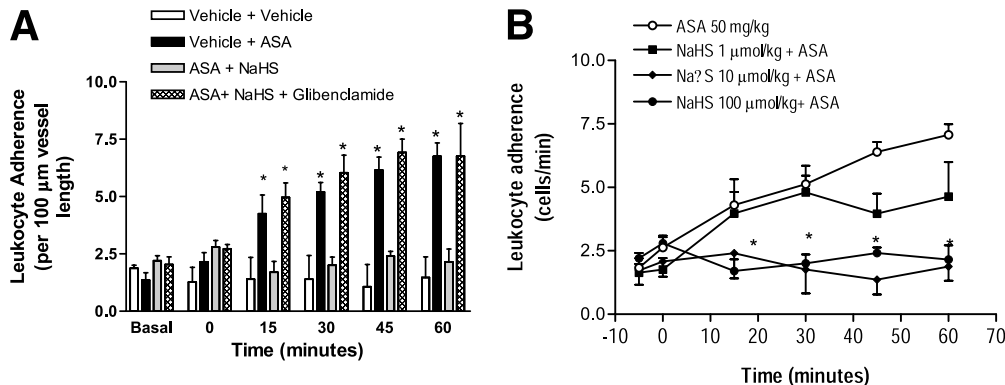


Figure 8. (A) Intra-gastric administration of ASA (50 mg/kg) increases leukocyte adherence to the vascular endothelium in mesenteric postcapillary venules. Cotreatment with NaHS (100 μmol/kg) attenuates ASA-induced leukocyte adherence. This effect was reversed by glibendamide, a K_{ATP} channel blocker. Data are expressed as mean ± SEM of 6 rats. *P < .05 vs vehicle of rats treated with ASA alone. (B) NaHS inhibits ASA-induced leukocyte adherence to mesenteric postcapillary venules in a dose-dependent manner. Data are expressed as mean ± SEM of 6 rats. *P < .05 vs vehicle of rats treated with ASA alone.

not the protein level, was attenuated after NSAID treatment, we speculated that an NSAID may modulate a specific intracellular signalling pathway to affect Sp1 function. Consistent with this view, we demonstrated that blockade of CSE expression by NSAIDs correlated with inhibition of ERK activity.

The ability of NSAIDs to regulate Sp1 phosphorylation might also help to explain COX-independent activities of ASA and NSAIDs. Abnormal Sp1 expression and activation have been observed in several human cancers including human hepatocellular carcinoma,³⁸ gastric carcinoma,^{39–41} and pancreatic adenocarcinoma.³⁸ Elevated

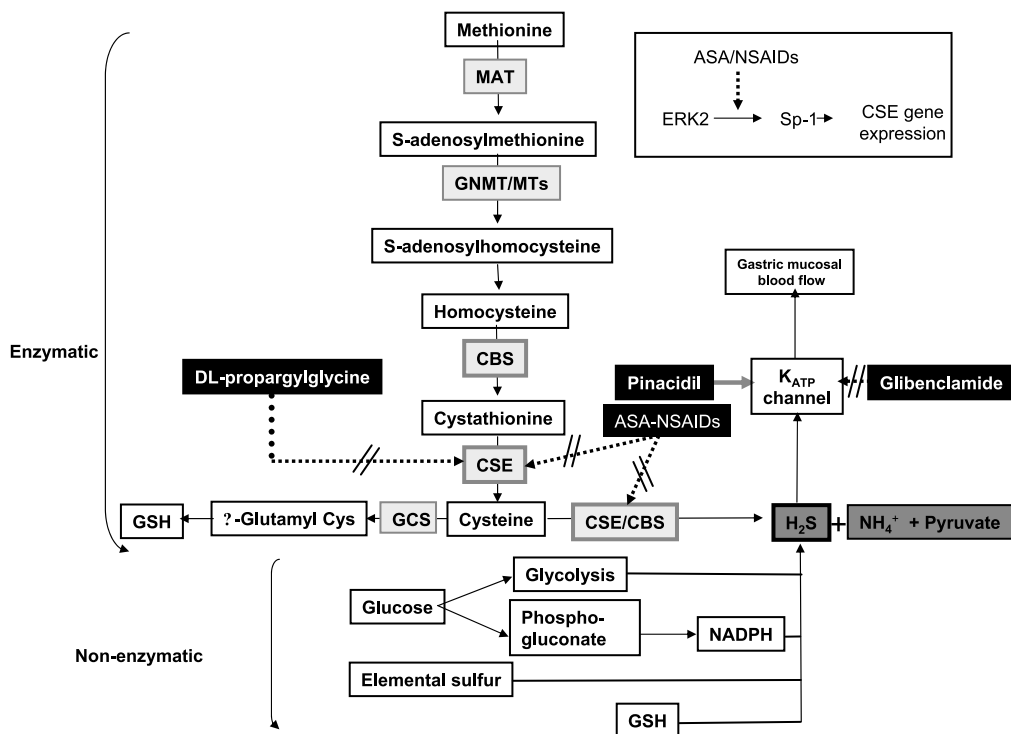


Figure 9. Role of H₂S in gastric mucosal protection. The scheme shows the major biochemical pathways involved in H₂S generation in mammals. Both the enzymatic pathway and nonenzymatic pathway of H₂S generation are shown. The enzymatic pathway is largely prevalent. DL-propargylglycine is a direct inhibitor of CSE activity. Glibendamide is a K_{ATP} channel blocker. Pinacidil is a K_{ATP} channel opener. Glibendamide and pinacidil exert divergent effects on gastric protection induced by H₂S. ASA and NSAIDs inhibit CSE expression/activity. MAT, methionine adenosyltransferase; GNMT, glycine N-methyltransferase; MTs, methyltransferases; CGS, γ-glutamyl-cysteine synthetase. *Inset:* Mechanism of CSE inhibition caused by ASA/NSAIDs. ASA/NSAIDs inhibit the ERK-dependent phosphorylation of Sp1, a key regulatory mediator of CSE gene expression.

Sp1 expression has also been shown to be correlated with malignancy and reduced survival of patients with gastric cancer.^{41,42} Because the CSE/H₂S pathway is also involved in regulating neoplastic growth,⁴³ our data suggest that inhibition of H₂S formation might contribute to the anticancer activity of ASA and NSAIDs.

Glibenclamide reversed the protection afforded by H₂S against the mucosal injury caused by ASA. Previous studies have shown that glibenclamide antagonized the protective effect of 16,16-dimethyl-PGE₂ on ethanol-induced gastric injury and exacerbated the mucosal injury induced by gastric perfusion with acidified ethanol, whereas pinacidil reverses the mucosal injury, suggesting that K_{ATP} channels mediate key steps in gastric mucosal homeostasis.^{44,45}

Similar to NaHS, L-cysteine and N-acetyl-cysteine were also effective in reducing gastric injury caused by ASA and NSAIDs. However, because these agents failed to increase gastric mucosal concentrations of H₂S, it appears that protection afforded by L-cysteine and N-acetyl-cysteine is H₂S independent. Indeed, in addition to release of H₂S, L-cysteine and N-acetyl-cysteine act as free-radical scavengers.⁴⁶

In conclusion, we have shown that H₂S regulates gastric mucosal blood flow and leukocyte adherence to the endothelium. These effects are likely mediated by K_{ATP} channels. Exposure to ASA/NSAIDs inhibits gastric expression/activity of CSE, a key enzyme for H₂S synthesis. By promoter deletion and mutation analysis, we demonstrated that NSAIDs act via the Sp1 transcription factor-binding site in the CSE promoter to suppress CSE gene expression. Together, these data establish a role for H₂S in gastric protection and identify CSE as a novel target for ASA/NSAIDs.

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