

Evidence That Hydrogen Sulfide Exerts Antinociceptive Effects in the Gastrointestinal Tract by Activating K_{ATP} Channels

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ABSTRACT

Hydrogen sulfide (H_2S) functions as a neuromodulator, but whether it modulates visceral perception and pain is unknown. Cystathionine β -synthase (CBS) and cystathionine- γ -lyase (CSE) mediate enzymatic generation of H_2S in mammalian cells. Here we have investigated the role of H_2S in modulating nociception to colorectal distension, a model that mimics some features of the irritable bowel syndrome. Four graded (0.4–1.6 ml of water) colorectal distensions (CRDs) were produced in conscious rats (healthy and postcolitic), and rectal nociception was assessed by measuring the behavioral response during CRD. Healthy rats were administered with sodium hydrogen sulfide (NaHS) (as a source of H_2S), L-cysteine, or vehicle. In a second model, we investigated nociception to CRD in rats recovering from a chemically induced acute colitis. We found that CBS and CSE are expressed in the colon and spinal cord.

Treating rats with NaHS resulted in a dose-dependent attenuation of CRD-induced nociception with the maximal effect at 60 μ mol/kg ($p < 0.05$). Administration of L-cysteine, a CSE/CBS substrate, reduced rectal sensitivity to CRD ($p < 0.05$). NaHS-induced antinociception was reversed by glibenclamide, a K^+ (K_{ATP}) channel inhibitor, and N^{ω} -nitro-L-arginine methyl ester hydrochloride (L-NAME), a nitric-oxide (NO) synthase inhibitor. The antinociceptive effect of NaHS was maintained during the resolution of colon inflammation induced by intrarectal administration of a chemical irritant. In summary, these data show that H_2S inhibits nociception induced by CRD in both healthy and postcolitic rats. This effect is mediated by K_{ATP} channels and NO. H_2S -releasing drugs might be beneficial in treating painful intestinal disorders.

Gaseous transmitters are a growing family of regulatory molecules involved in regulation of physiological and pathological functions in mammalian tissues (Wang, 2002; Boehning and Snyder, 2003). Whereas nitric oxide (NO) is the best characterized member of this family, it is increasingly recognized that carbon monoxide (CO) and hydrogen sulfide (H_2S) also exert regulatory functions. H_2S is endogenously generated from L-cysteine through the activity of two pyridoxal-5'-phosphate-dependent enzymes, the cystathionine γ -lyase (CSE) and cystathionine β -synthase (CBS), although alternative sources (e.g., by activity of cysteine aminotransferase and/or 3-mercaptosulfotransferase) cannot yet be discounted (Wang 2002; Boehning and Snyder, 2003; Moore et al., 2003).

In some tissues, CSE and CBS are both needed for generation of H_2S , whereas in others one enzyme suffices. The expression of CBS and CSE has been identified in several mammalian tissues, including liver, kidney, brain, ileum, and blood lymphocytes. In the cardiovascular system, H_2S , mostly derived from CSE, modulates endothelium-dependent and endothelium-independent vasodilatation (Zhao et al., 2001; Wang, 2002), whereas CBS-derived H_2S is a physiologically relevant neuromodulator in the central nervous system (CNS) (Wang, 2002; Boehning and Snyder, 2003). Consistent with this view, it has been shown that H_2S is present at relatively high levels in the mammalian brain and that, in the CNS, the activity of CBS is >30-fold greater than that of CSE (Awata et al., 1995). In addition, the reduced H_2S production after inhibition of CBS and the fact that CSE inhibitors do not suppress H_2S production in the CNS further

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ABBREVIATIONS: NO, nitric oxide; H_2S , hydrogen sulfide; CBS, cystathionine β -synthase; CSE, cystathionine- γ -lyase; CRD, colorectal distension; MPO, myeloperoxidase; NaHS, sodium hydrogen sulfide; L-NAME, N^{ω} -nitro-L-arginine methyl ester hydrochloride; IBS, irritable bowel syndrome; AWR, abdominal withdrawal reflex; TNBS, trinitrobenzene sulfonic acid; SAOB, sulfide antioxidant buffer; RT, reverse transcription; PCR, polymerase chain reaction.

pinpoint CBS to be the major H₂S-producing enzyme in neural tissues (Abe and Kimura, 1996).

H₂S regulates key neuronal functions, including the induction of hippocampal long-term potentiation, a synaptic model of learning and memory (Abe and Kimura, 1996; Kimura, 2000), and the release of the corticotrophin-releasing hormone from the hypothalamus (Russo et al., 2000). Although the molecular mechanisms involved in these activities are only partially known, it has been shown that H₂S increases cAMP levels in neuronal and glial cell lines and primary neuron cultures and hyperpolarizes dorsal raphe neurons by activating the ATP-sensitive K⁺ (K_{ATP}) channels. In addition, H₂S causes a cAMP-dependent potentiation of *N*-methyl-D-aspartate receptors (Moore et al., 2003). Previous studies have shown that, at low concentrations, H₂S enhances the smooth muscle relaxation effect of NO, suggesting that a “cross-talk” between the two gases exists (Hosoki et al., 1997). Furthermore, the NO donor sodium nitroprusside enhances brain CBS activity *in vitro* (Eto and Kimura, 2002).

It has been demonstrated that minimal inflammatory changes in the colon are associated with irritable bowel syndrome (IBS) (Collins et al., 2001), a clinical disorder linked with an altered cortical integration of painful messages and a hyperalgesic response to colorectal distension (CRD). Several mediators, including NO, have been implicated in the transmission of visceral noxious and non-noxious sensations to CNS. Whether H₂S modulates visceral nociception during CRD is still unknown.

In this study, we have investigated the effects of H₂S administration in rodent models of visceral nociception. Our results demonstrate that H₂S modulates nociception induced by CRD in healthy and colitic rats, providing the ground for the development of H₂S-based therapy in the treatment of painful abdominal condition in humans.

Materials and Methods

Materials. Sodium hydrogen sulfide (NaHS), L-cysteine, glibenclamide, pinacidil, L-NAME, ascorbic acid, salicylic acid, potassium hydroxide, *N*-acetyl-L-cysteine, DL-propargylglycine, trichloroacetic acid, pyridoxal-5'-phosphate, and calmodulin were from Sigma-Aldrich (St. Louis, MO). The stock solution of NaHS was freshly prepared by dissolving NaHS immediately before use. Tissue protein extraction reagent was obtained by Pierce Biotechnology (Rockford, IL). All of the chemicals were of analytical grade and were used without treatment. Deionized filtered water was used for the buffer preparation. Silver and sulfide ion-selective electrode was from ThermoOrion (Beverly, MA).

Animals. Male Wistar rats (200–250 g; Charles River Italice, Calco, Italy) were housed in plastic cages and maintained under controlled conditions with 12-h light/dark cycles with lights on at 7:00 AM. Tap water and standard laboratory chow were freely available. Food was withheld for 12 h before surgical procedures and CRD recordings. After recovery from surgery, the rats were individually trained by spending 2 to 3 h per day in a Plexiglas cage for 2 to 3 days. It allowed them to adjust to a movement-restriction environment. All experimental procedures described below were approved by our institutional animal research committees and were in accordance with nationally approved guidelines for the treatment of laboratory animals. All experiments were performed in conscious, unanesthetized rats and were conducted in a blind manner, in that the observer was not aware of the identity or dose of drugs administered to each animal.

Surgical Procedures. Fasting rats were anesthetized with pentobarbital (60 mg/kg *i.p.*), and a catheter was inserted into the left

jugular vein. The catheter was externalized subcutaneously through the dorsal aspect of the neck and protected with a tube attached to the skin for future access. During procedure, body temperature was kept constant at 36–37°C using a homeothermic blanket. Animals exhibiting motor deficits after the surgical procedure were not used in the experiment. Following surgery, rats were housed separately and were allowed to recuperate for at least 5 days before CRD testing. Rats were allowed to recover from the surgical procedure for 3 days before subsequent training in the Plexiglas cage.

CRD and Behavioral Testing. The night before experiments, the balloons were inflated and left overnight so that the latex stretched and the balloons became compliant. On the testing day, each rat was sedated with ether inhalation, and a 2-cm long latex balloon was inserted intrarectally 2 cm from the anal verge and fixed at the base of the tail. The balloon was connected via a double-barreled cannula to a pressure transducer continuously monitoring the colorectal pressure by a computer (PowerLab PC; A.D. Instruments, Milford, MA) and to a syringe for inflation/deflation of the balloon. The rats were then housed in a small Plexiglas cage (20 × 8 × 8 cm) on an elevated platform and were allowed to regain consciousness and adapt for 1 h. After recovery from sedation, the rats underwent the CRD procedure and behavioral response was tested in all groups, with the exception of the control group in which no CRD was performed. CRD of 20 s performed every 5 min was applied in increments of 0.4 ml starting from 0.4 ml and increasing to 1.6 ml of water. To achieve an accurate measurement of the colonic parameters and perception, each distension was repeated twice and data were averaged for analysis. Animals underwent double sets of CRD. Ten minutes after the first CRD (0.4–1.6 ml of water), drugs were administered *i.p.* and/or *i.v.* Five minutes after the end of the drugs administration, a second CRD was performed. Behavioral responses and colonic parameters collected during the first and second sets of CRD were assessed and compared.

The behavioral response to CRD was assessed by measuring the abdominal withdrawal reflex (AWR) using a semiquantitative scoring system (Al-Chaer et al., 2000). The AWR is an involuntary motor reflex similar to the visceromotor reflex, but it has the great advantage that the latter requires abdominal surgery to implant recording electrodes and wires in the abdominal muscle wall, which may cause additional sensitization (Ness and Gebhart, 1990). Measurement of the AWR consisted of visual observation of the rat's response to graded CRD by blinded observer and assignment of an AWR score according with the behavioral scale described previously (Al-Chaer et al., 2000) in which grade 0 corresponds to no behavioral response to CRD, grade 1 corresponds to brief head movement at the onset of the stimulus followed by immobility, grade 2 corresponds to a mild contraction of abdominal muscles, although the rat does not lift the abdomen off the platform, grade 3 corresponds to a strong contraction of the abdominal muscles with the lifting of the abdomen off the platform, and grade 4 corresponds to a severe contraction of the abdominal muscles manifested by body arching and the lifting of the abdomen and of the pelvic structures and scrotum. The rats that did not show a behavioral response (i.e., score 0) were excluded (~20%). To determine the effect of H₂S on colonic smooth muscle, the compliance of the colon during CRD was obtained from colorectal volume and pressure and expressed as milliliter/mm Hg.

Effects of H₂S on Colonic Nociception. The control group (*n* = 5) consisted of fasting rats that underwent surgical procedures but not CRD. To investigate whether H₂S administration modulates sensitivity and pain induced by CRD, rats were treated *i.p.* with NaHS (as H₂S donor) at doses of 15, 30, or 60 μmol/kg (NaHS group), L-cysteine (the natural substrate for H₂S formation) at the dose of 100 μmol/kg (L-cysteine group), or vehicle (CRD group). NaHS was diluted in a 1% methylcellulose medium. In these experiments, NaHS was used as H₂S donor for the following reasons: 1) NaHS dissociates to Na⁺ and HS⁻ in solution, and then HS⁻ associates with H⁺ and produces H₂S. At physiological pH, approximately one-third of the H₂S exists as the undissociated form (H₂S), whereas

the remaining two-thirds is HS⁻ at equilibrium with H₂S (Beauchamp et al., 1984); 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 (<1 mM) is negligible; 4) NaHS at concentrations used in the present study does not change the pH of the medium. For these reasons, NaHS has been widely used for studies of H₂S.

The involvement of K_{ATP} channels in the modulation of visceral perception by H₂S was assessed by pretreating rats with glibenclamide (a K_{ATP} channel blocker) at a dose of 2.8 μmol/kg i.v. for 20 min before NaHS (60 μmol/kg i.p.) administration (glibenclamide + NaHS group) or glibenclamide alone (glibenclamide group). To confirm that the effects of H₂S on visceral perception are mediated by an action on the K_{ATP} channels, pinacidil (a K_{ATP} channels opener) at the dose of 2.8 μmol/kg i.v. was administered for 20 min between the two CRD sets (pinacidil group). To investigate whether NO is involved in the H₂S-mediated effects on visceral nociception, L-NAME, a nonselective NO synthase inhibitor, was infused i.v. at the dose of 100 μmol/kg for 20 min before NaHS (60 μmol/kg i.p.) administration (L-NAME + NaHS group). At the end of the CRD procedures, rats were sacrificed and blood, colon, and spinal cord (L1–L5) were removed and collected for further analysis.

Induction of Colitis. Colitis was induced as described previously (Fiorucci et al., 2002). In brief, rats (14 animals) were anesthetized with pentobarbital (60 mg/kg i.p.) and trinitrobenzene sulfonic acid (TNBS) at the dose of 20 mg/ml in 0.5 ml of 50% ethanol was administered into the distal colon by cannula. The rats were monitored daily for loss of body weight and survival. After 2 weeks, animals that were still alive underwent CRD study as described above. In the first group, we performed two consecutive series of CRD (TNBS + CRD group), whereas in the second group, CRD was repeated after treatment with NaHS at the dose of 60 μmol/kg i.p. (TNBS + CRD + NaHS group). At the end of the CRD procedures, rats were sacrificed and blood, colon, and spinal cord were taken and collected for further analysis.

Assessment of Colonic Inflammation. Colons were examined with a dissecting microscope (5-fold magnification) and graded for macroscopic lesions on a scale from 0 to 10 based on criteria for inflammation, such as hyperemia, thickening of the bowel, and the extent of ulceration (Wallace et al., 1989). Colonic tissue was taken for MPO activity assessment, an index of granulocyte infiltration into the tissue, as described previously (Santucci et al., 1995).

Measurement of Plasma H₂S Concentration and H₂S Production. Plasma H₂S concentrations and enzymatic capacity for H₂S production in colon and spinal cord were measured as described previously (Hosoki et al., 1997; Zhao et al., 2001; Ubuka, 2002) with modifications. In brief, 250 μl of plasma were added to ice-cold 250 μl of NaOH (0.5 N) in a sealed three-neck reactor. A constant stream of nitrogen was passed through the mixture via gas-inlet capillary. The reactor was maintained at 37°C, and H₂S extraction was started by introducing 1 ml of 10% trichloroacetic acid solution. The stream of nitrogen carried the sulfide acid in another reactor by cooled connector and bubbling in 2 ml of sulfide antioxidant buffer (SAOB) solution, consisting of 2 M KOH, 1 M salicylic acid, and 0.22 M ascorbic acid at pH 12.8. After 30 min, the SAOB solution was removed, and the sulfide concentration was measured with a sulfide-sensitive electrode (model 9616 S²⁻/Ag⁺ electrode; Thermo Electron Corporation, Waltham, MA) and expressed as H₂S (Khan et al., 1980; Ubuka, 2002).

One hundred milligrams of colon or spinal cord samples were homogenized in 1 ml of ice-cold tissue protein extraction reagent protein extractor. The enzymatic capacity for H₂S production was performed on the same reactor as for the plasma analysis. Two milliliters of an assay reaction mixture was introduced in the reactor. The mixture contained 10 mM L-cysteine, 2 mM pyridoxal 5'-phosphate, 100 mM potassium phosphate buffer (pH 7.4), and 20% (w/v) colonic or spinal cord homogenate. A constant stream of nitrogen was passed through the mixture via gas-inlet capillary. Reac-

tions were initiated by transferring the tube from ice bath to a 37°C water bath. The stream of nitrogen carried the sulfide acid in the second reactor containing 4 ml of SAOB as described previously (Eto and Kimura, 2002). After incubating at 37°C for 90 min, 1 ml of 50% trichloroacetic acid solution was added to the mixture to stop the reaction. The remainder H₂S in the mixture was carried out via nitrogen stream by other 30 min of incubation at 37°C. The concentration of sulfide in SAOB solution was measured with a sulfide-sensitive electrode as described previously (Eto and Kimura, 2002).

Colonic and Spinal CBS and CSE and Spinal c-Fos Expression. Total RNA was isolated from rat colon and spinal cord by using the TRIzol reagent according to manufacturer's specifications (Invitrogen, Carlsbad, CA). RNA was processed directly to cDNA by reverse transcription with Superscript II (Invitrogen). In brief, 2 μg of RNA was added to mixture that contained DNase I reaction buffer (10×) and 1 U of DNase I. The mixture was incubated for 15 min at room temperature; then 4 μl of 5× first-strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, and 15 mM MgCl₂), 2 μl of DDT (0.1 M), 2 μl of dNTP mixture (10 mM), 1 μl of random primers (300 ng/μl), 0.5 μl of RNase, and 0.5 μl of SuperScript II were added to the sample. The mixture was incubated at room temperature for 10 min and at 42°C for 50 min, heated at 95°C for 5 min 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 (Ebersberg, Germany). For rat CBS, the sense primer was 5'-CCAGGACTTGGAGGTACAGC-3' and the antisense primer was 5'-TCGGCACTGTGTGGTAATGT-3'; for rat CSE, the sense primer was 5'-GTATTGAGGCACCAACAGGT-3' and the antisense primer was 5'-GTTGGGTTTGTGGGTGTTTC-3'; for the rat c-Fos, the sense primer was 5'-GTCTGGTTCCTTCTATG-CAG-3' and the antisense primer was 5'-AGGTAGTGCAGCTGG-GAGT-3'. In control experiments with three replicates, no false positive were detected. Amplification reactions contained 2 μl of cDNA, 12.5 μl of the 2× Dynamo SYBR Green qPCR Master Mix, and 0.75 μl of each of the specific primers (30 μM). Primer concentrations in the final volume of 25 μl were 300 nM. All reactions were performed in triplicate in an iCycler iQ system (Bio-Rad, Hercules, CA), and thermal cycling conditions were 15 min at 95°C followed by 40 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 20 s.

Statistical Analysis. All data are presented as the mean ± S.E.M., with sample sizes of at least five rats/group; statistical comparisons of unpaired data were performed by the Mann-Whitney test, whereas statistical comparisons of paired data were performed by the Wilcoxon signed rank test. An associated probability (*p* value) of less than 5% was considered significant.

Results

CBS and CSE Are Expressed in the Colonic Tissue and Spinal Cord. As illustrated in Fig. 1A, mRNAs encoding for CBS and CSE, the two key enzymes involved in H₂S formation from L-cysteine, were expressed in rat colonic and spinal cord tissues. Incubating colonic and spinal cord homogenates with L-cysteine, the natural substrate of CBS and CSE, in the presence of pyridoxal-5'-phosphate, an essential factor for enzyme activities, resulted in H₂S generation (Fig. 1B, left side). In the presence of L-cysteine, the colon generated ≈2 nmol/min/g protein of gaseous H₂S, whereas spinal cord homogenates incubated at the same conditions produced ≈6 nmol/min/g protein of gaseous H₂S. Plasma H₂S concentration was ≈50 μM, similar to that reported previously by others (Guidotti 1996). The amounts of H₂S detected in the rat colon and spinal cord did not change during CRD alone or after NaHS or L-cysteine administration (data not shown).

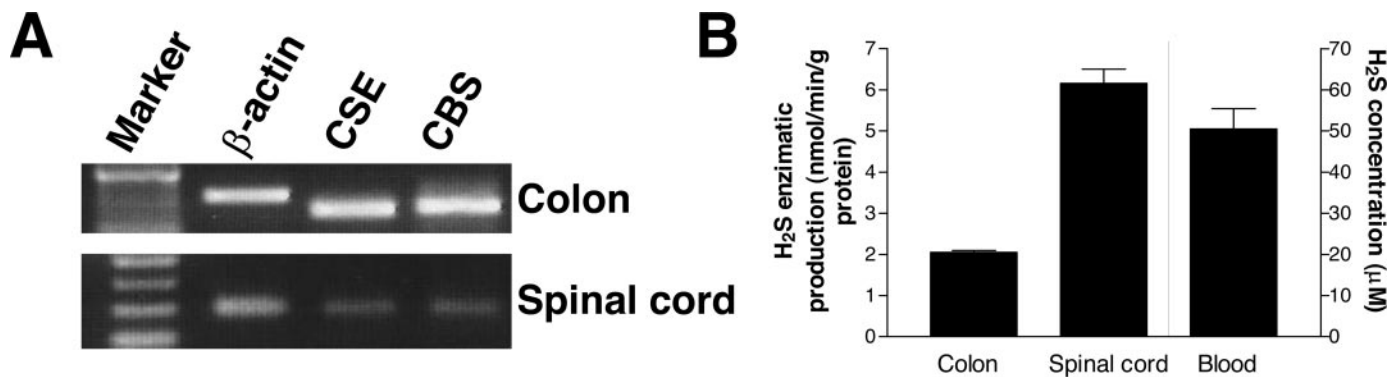


Fig. 1. CBS and CSE are expressed in the colon and spinal cord. A, RT-PCR expression of CSE and CBS in the colon (above) and spinal cord (below). The RT-PCR shown is representative of four. B, endogenous production of H₂S by colon and spinal cord homogenates and physiological plasmatic concentrations of H₂S.

H₂S Inhibits CRD-Induced Nociception. In all subsequent experiments, two sequential distension-effect curves were constructed. The first distension-effect curve acted as basal, and the second curve was constructed following saline or drugs administration. In all experiments, all of the animals were conscious and we observed that any drugs, including NaHS, did not induce changes in the state of consciousness. CRD (0.4–1.6 ml of water) elicited volume-dependent increases in the AWR score, which were rapid in onset and persisted for the duration of the distension period (Fig. 2A) with no significant reduction in colorectal pressure (Fig. 2B). Distensions with 0.4 ml of water induced a slight increment of the AWR score (<1) that was associated with a small rise of colorectal pressure (\approx 20 mm Hg), indicating that this CRD represents a nonpainful stimulus, whereas distensions with 1.2 and 1.6 ml of water were related to the maximal AWR scores (3 and 4, respectively) and to very high colorectal pressures (up to 80 mm Hg), indicating that these volumes induce noxious sensations (Ji and Traub, 1991).

Injected i.p., NaHS caused a dose-dependent reduction of the AWR score. At the lower dose (15 μ mol/kg), there was no effect on AWR score (Fig. 2C) or colorectal compliance (Fig. 2D), whereas at doses of 30 and 60 μ mol/kg, NaHS significantly reduced the CRD-induced AWR at all volumes of distension applied (Fig. 2, E and G, respectively). The decrease in the AWR response was associated to a decrease in rectal compliance only with the higher dose of NaHS (Fig. 2H), suggesting that, at the lower doses (15–30 μ mol/kg), NaHS failed to induce colonic smooth muscle relaxation (Fig. 2, D and F, respectively).

Similarly to NaHS, intraperitoneal administration of L-cysteine (100 μ mol/kg i.p.) caused a significant decrement of the AWR response to CRD (Fig. 3A) with a concomitant increase in rectal compliance ($P < 0.05$ versus CRD group) (Fig. 3B). This effect was reverted by pretreating rats with DL-propargylglycine (100 μ mol/kg i.p.) (Fig. 3, C and D).

Data on the antinociceptive effects of H₂S during CRD were confirmed by analysis of c-Fos expression in the spinal cord. Quantitative RT-PCR of c-FOS mRNA expression demonstrates that CRD induced a 2-fold increase in spinal c-Fos expression ($n = 5$; $p < 0.05$ versus control), confirming its nociceptive action (Fig. 4). Administration with either NaHS or L-cysteine abrogated c-FOS mRNA induction caused by CRD, indicating that the reduced AWR score was due to the antinociceptive effect of H₂S rather than a possible effect of

this gas on the consciousness state ($n = 5$; $p < 0.05$ versus CRD). Basal plasma concentrations of H₂S were \approx 47 μ M and did not change during CRD or following treatment with NaHS or L-cysteine (data not shown).

K_{ATP} Channels Inhibition Reverts Antinociceptive Effect of H₂S. To determine whether or not ATP-sensitive K⁺ channels were involved in the antinociceptive effect of H₂S, the interaction of H₂S with known K_{ATP} channel modulators was examined. The inhibitory effect of NaHS on CRD-induced pain was completely reversed by pretreating rats with 2.8 μ mol/kg glibenclamide, a K_{ATP} channel antagonist (Fig. 5A). Moreover, glibenclamide inhibited colonic smooth muscle relaxation-induced by the highest dose of NaHS (Fig. 5B). The antinociceptive and relaxant effects of NaHS were mimicked by the K_{ATP} channel opener pinacidil (Fig. 5, C and D). In contrast, treating rats with glibenclamide alone had no effect on CRD-induced nociception or colonic compliance (Fig. 5, E and F, respectively). Analysis of c-Fos expression confirmed the colorectal nociception and pain data. As illustrated in Fig. 5G, pretreatment with glibenclamide had no effect, whereas pinacidil down-regulated the c-Fos mRNA expression induced by CRD. Plasma H₂S concentrations did not significantly change during K_{ATP} channel modulators treatments (data not shown).

NO Synthase Inhibition Reverts Antinociceptive Effect of H₂S. Pretreating rats with L-NAME at the dose of 100 μ mol/kg i.v. 10 min before NaHS administration (60 μ mol/kg i.p.) produced an almost complete inhibition of the antinociceptive (Fig. 6A) and relaxant (Fig. 6B) effects of NaHS. Consistent with the reversal of the antinociceptive effect of NaHS by L-NAME, spinal c-Fos expression was significantly up-regulated during L-NAME pretreatment (Fig. 6C). L-NAME administration reduced the plasma concentrations of H₂S when compared with those of control animals and other treatments (Fig. 6D).

Colonic Damage and MPO Activity. Macroscopic examination of the colon revealed that the inflammation scores after CRD alone or CRD plus drugs administration were similar to those of control group (data not shown). Moreover, MPO activity in colonic tissue during CRD was similar to that of the control group, indicating that CRD did not produce a significant colonic inflammatory response. Administration of NaHS and L-cysteine, glibenclamide, and L-NAME groups did not significantly affect colonic MPO activity (data not shown).

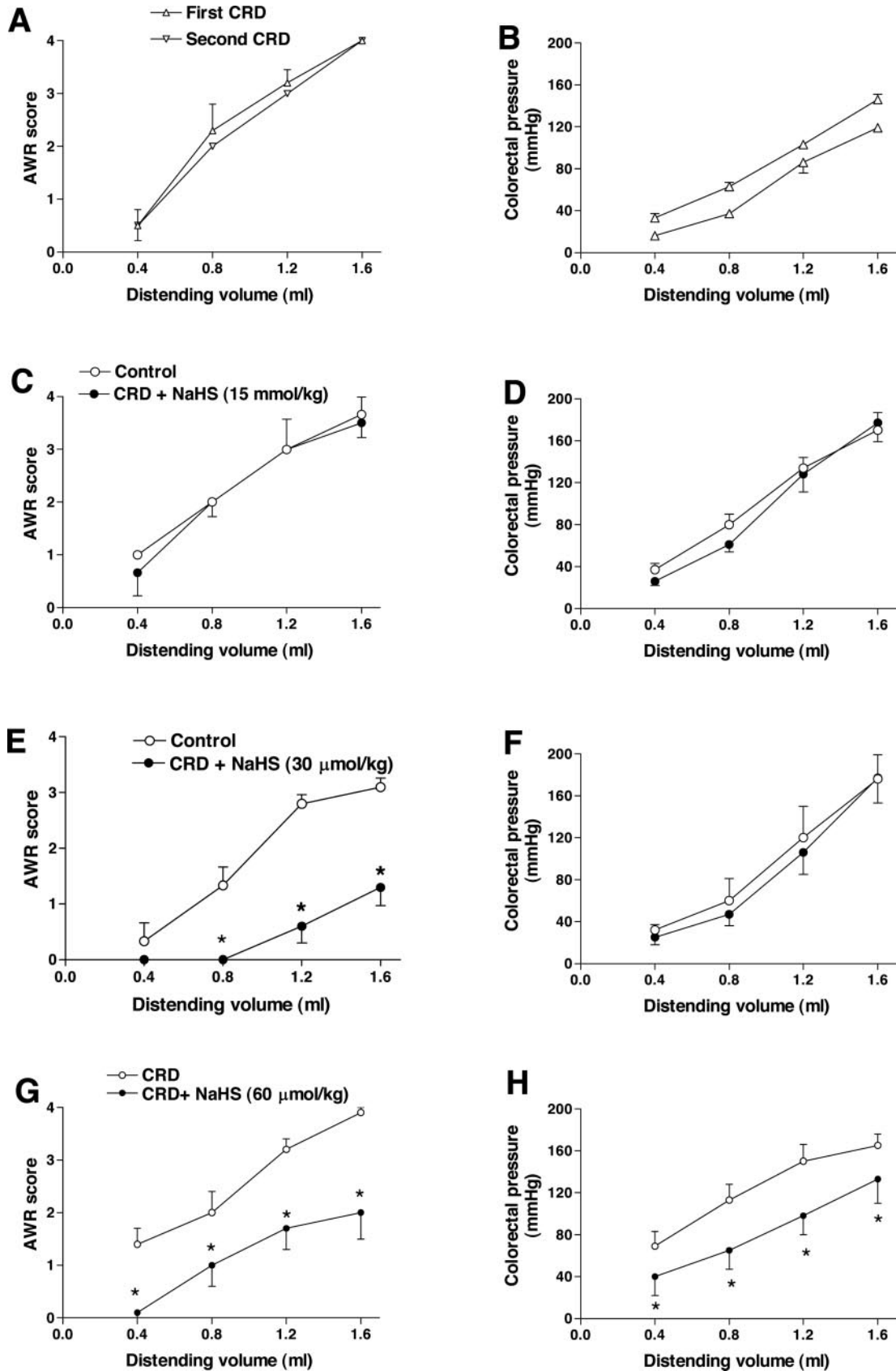


Fig. 2. H₂S reverses CRD-induced nociception in conscious rats. CRD induces a volume-dependent, reproducible increment of the AWR (A) without a significant change of colorectal compliance (B). NaHS administration determines a dose-dependent inhibition of the nociceptive action of the CRD that is significant at the doses of 30 (E) and 60 μmol/kg (G), whereas the dose of 15 μmol/kg is ineffective (C). At the doses of 15 (D) and 30 μmol/kg (F), no change of the colorectal compliance is observed, whereas at the higher dose, NaHS induces a significant colorectal relaxation (H). Data are mean ± S.E. of five rats. *, *p* < 0.05 versus control.

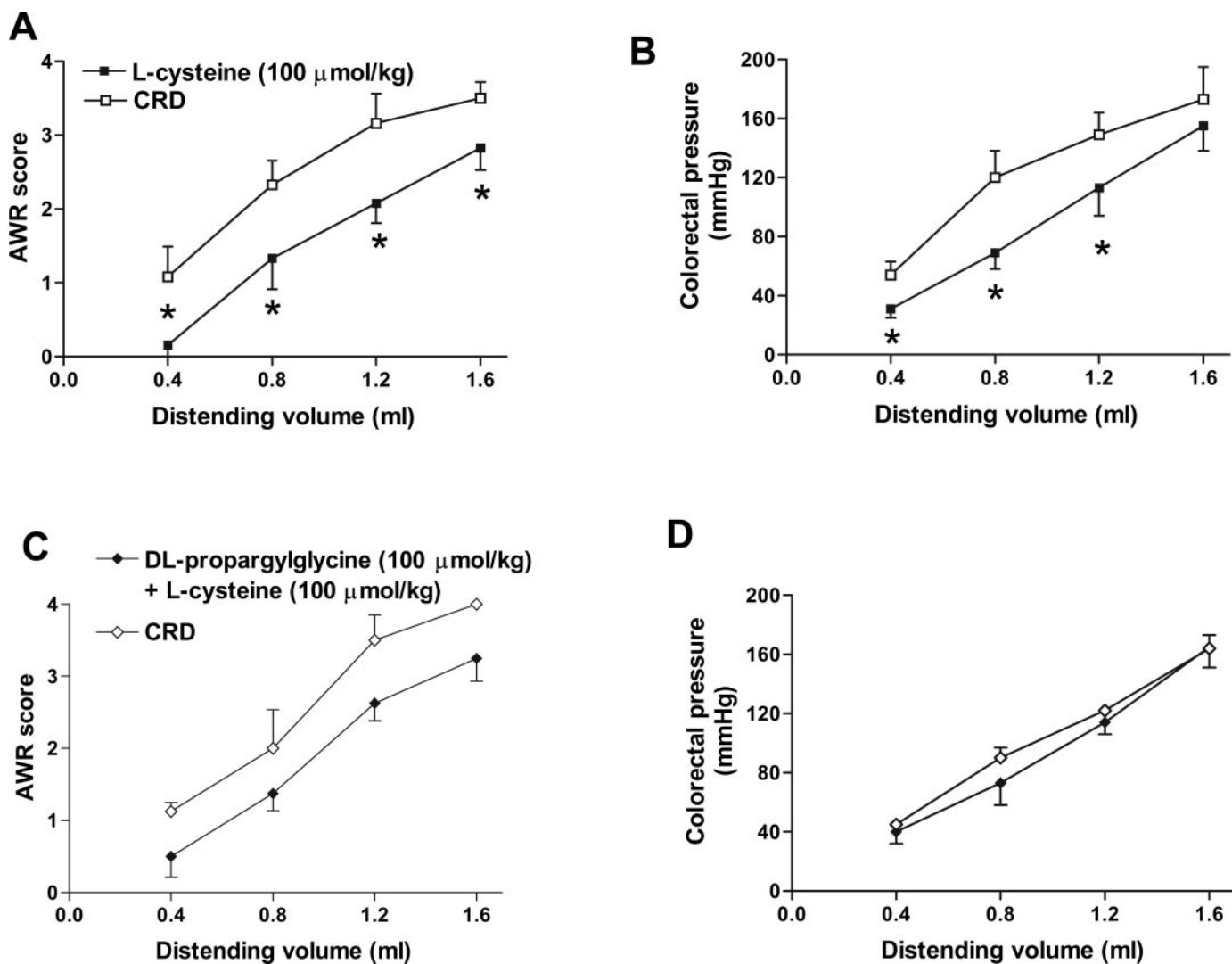


Fig. 3. L-Cysteine reproduces the effects of H_2S . L-Cysteine causes a significant reduction of the AWR score (A) and relaxation of the colorectal muscular wall as demonstrated by the increment of the colorectal compliance (B). DL-Propargylglycine, a CSE inhibitor, reverses antinociceptive (C) and relaxant (D) effects of L-cysteine. Data are mean \pm S.E. of five rats. *, $p < 0.05$ versus control.

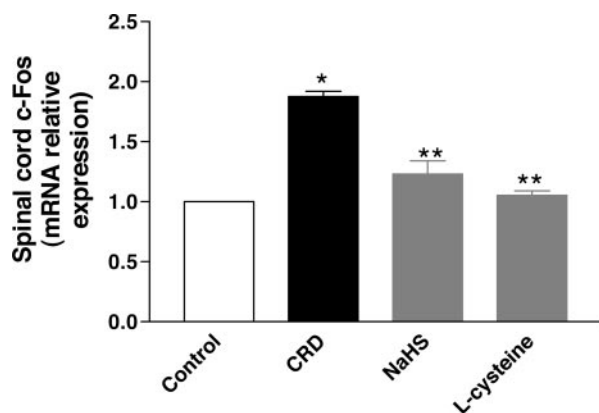


Fig. 4. H_2S inhibits the expression of c-Fos mRNA in the spinal cord. CRD induces an overexpression of spinal c-Fos mRNA that is reversed by NaHS and L-cysteine administration. Data are mean \pm S.E. of five rats. *, $p < 0.05$ versus control. **, $p < 0.05$ versus CRD alone.

H_2S Inhibits Pain in Inflamed Rats. The rats with colitis exhibited a loss of weight of approximately 20% when compared with healthy rats, and diarrhea was observed dur-

ing the first week after induction of colitis. Macroscopic inflammation and thickening of bowel wall was observed in TNBS-treated rats compared with controls, whereas hyperemia and ulceration were largely resolved. Confirming the presence of inflammation MPO activity was also significantly increased in TNBS-treated rats in comparison with controls (data not shown). However, there was no difference in macroscopic score or MPO activity between the two groups of rats with colitis. When CRD was performed two weeks after induction of colitis, a significant increase in the AWR score was observed in comparison with that control in rats. As shown in Fig. 7A, an increased nociception was observed during the low volume distensions (0.4 and 0.8 ml of water), indicating that colonic inflammation induces allodynia (perception of nonpainful stimulus as painful) and hyperalgesia (perception of painful stimulus as more painful) to CRD. The AWR score determined during repeated CRD did not change, whereas pretreating colitic rats with NaHS (60 μ mol/kg i.p.) almost completely inhibited the allodynic response to CRD (Fig. 7B). These data were confirmed by the analysis of spinal c-Fos mRNA. The expression of c-Fos mRNA in the spinal cord was

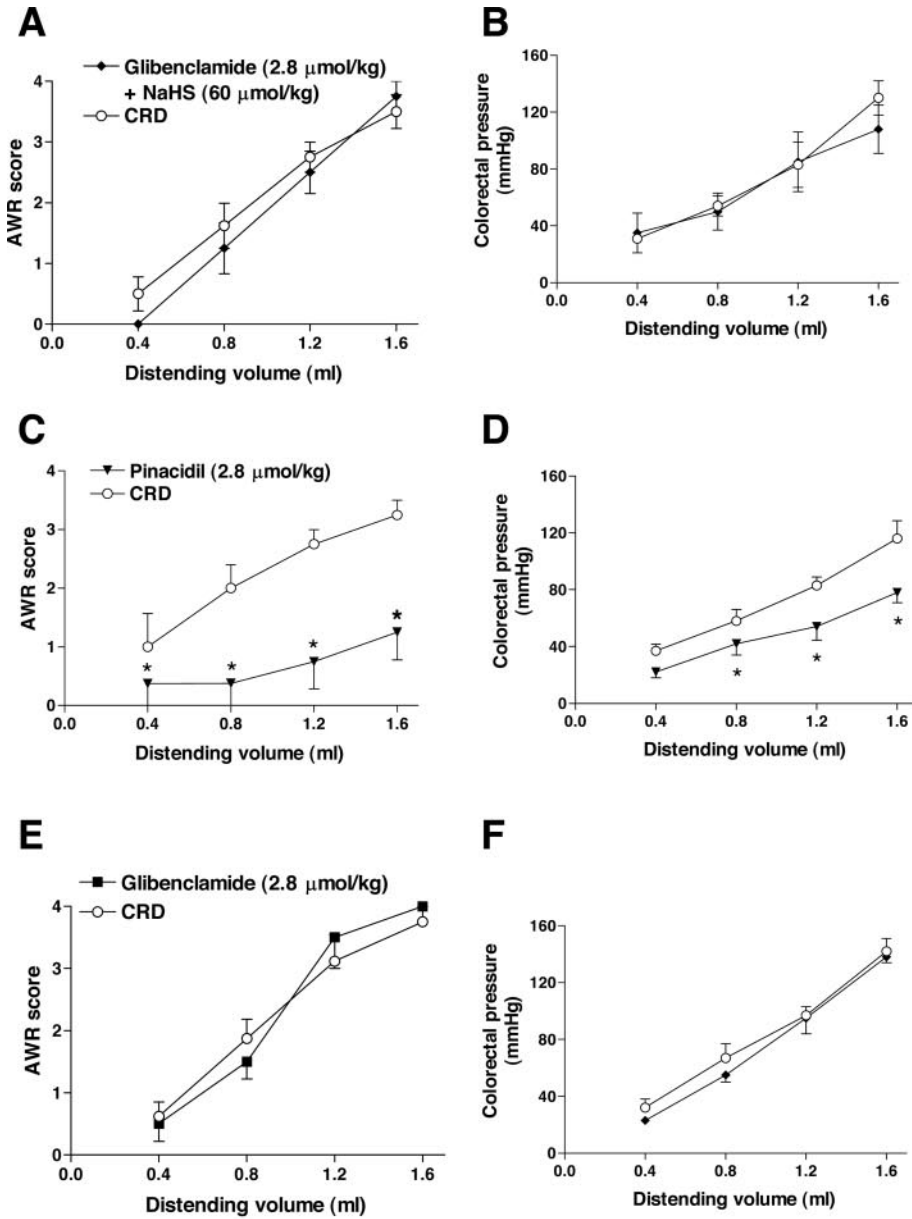
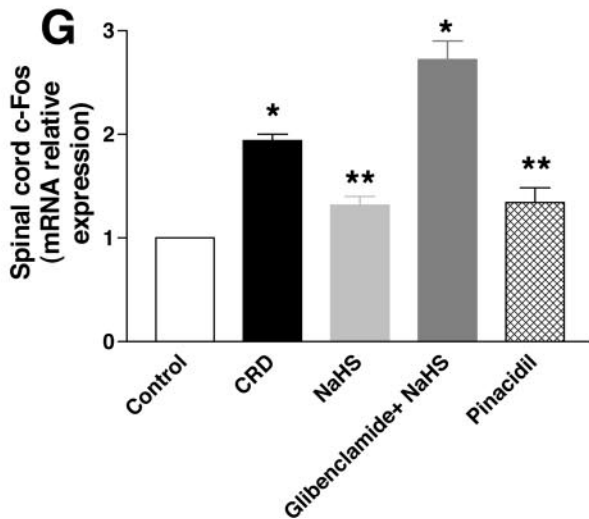


Fig. 5. The antinociceptive effect of H₂S is modulated by K_{ATP} channels. Pretreating rats with the K_{ATP} channel blocker glibenclamide abrogates the antinociceptive (A) and myorelaxant (B) effects of NaHS, whereas the K_{ATP} channel opener pinacidil reproduces the effects of NaHS on both the visceral sensitivity (C) and the colorectal compliance (D). Glibenclamide alone does not induce any changes of the AWR score (E) and the colorectal compliance (F). These results are confirmed by quantitative RT-PCR data on spinal c-Fos expression (G). Data are mean ± S.E. of five rats. *, *p* < 0.05 versus control. **, *p* < 0.05 versus CRD alone.



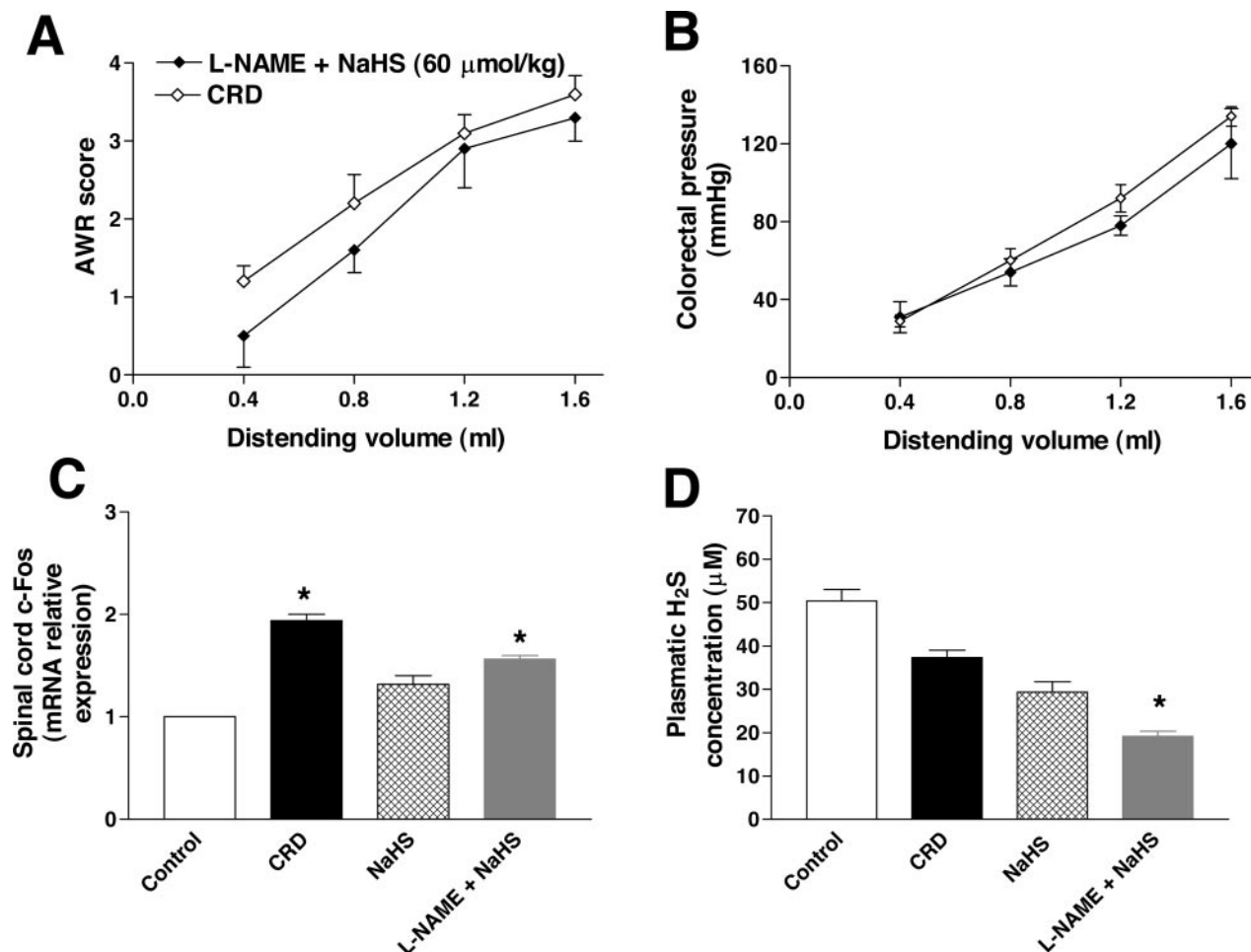


Fig. 6. The antinociceptive effect of NaHS is modulated by NO. Pretreating rats with the NOS inhibitor L-NAME abrogates the antinociceptive (A) and myorelaxant (B) effects of NaHS. Quantitative RT-PCR analysis of c-Fos mRNA expression in the spinal cord confirms the functional data (C). L-NAME administration reduces plasmatic H₂S concentration (D). Data are mean \pm S.E. of five rats. *, $p < 0.05$ versus control.

greatly increased in the colitic rats before and after CRD, indicating the presence of a painful condition after induction of colitis. The administration of NaHS reduced c-FOS mRNA expression to values similar to that of controls (Fig. 7C).

Discussion

The studies described herein demonstrate that H₂S functions as a negative regulator of visceral nociception by activating K_{ATP} channels and NO-dependent mechanisms and attenuates pain in a rodent model of colonic inflammation. The key finding of this study was the demonstration that H₂S inhibits CRD-induced nociception in rats. At the lower dose (15 μmol/kg), NaHS did not cause any change in behavioral response in conscious rats, whereas at higher doses (30 and 60 μmol/kg), it significantly decreases the AWR score following repetitive noxious (1.6 ml) and non-noxious (0.4 ml) CRD. Several mechanisms might explain the antinociceptive effect of H₂S. First, H₂S is a potentially toxic gas whose major lethal consequence is the loss of central respiratory drive due to biochemical lesions of the respiratory centers of the brainstem (Khan et al., 1980). Thus, in theory, H₂S may blunt sensorial functions, causing a loss of consciousness that mimics a pain-free condition during CRD. However, this is an unlikely explanation because we did not observe any change

in the consciousness during these studies. In addition, endogenously generated H₂S is rarely accumulated or toxic to cells due to the cellular metabolism of the gas. Finally, concentrations of $<30 \mu\text{M}$ H₂S cause no apparent disturbance in oxidative phosphorylation because of the rapid oxidation of H₂S in mitochondria (Guidotti, 1996). Recent data demonstrated that physiologic plasma H₂S concentrations in rats are $\approx 46 \mu\text{M}$ (Calderone et al., 1996). In the present study, the plasma concentrations of H₂S ranged from 30 to 55 μM, unlikely making it a central toxic effect of the gas. A second explanation is that H₂S alters the compliance of the colorectum. Human studies (Distrutti et al., 2004) have shown that perception of intestinal distension depends on the state of rectal tone. When the rectum is relaxed (in both physiological and pharmacological conditions), perception of rectal distension decreases, and many pharmacological approaches have been tested (particularly in IBS patients) to reduce the rectal tone by manipulating the properties of the smooth muscle tone of the intestinal wall. In our experiments, we found that H₂S significantly inhibited CRD-induced nociception at the doses of 30 and 60 μmol/kg, but increased rectal compliance only at the higher dose. Thus, whereas H₂S has been shown to modulate smooth muscle tone, we have provided evidence that modulation of pain perception is not directly related to its effect on colon tone and reduction of nociception is ob-

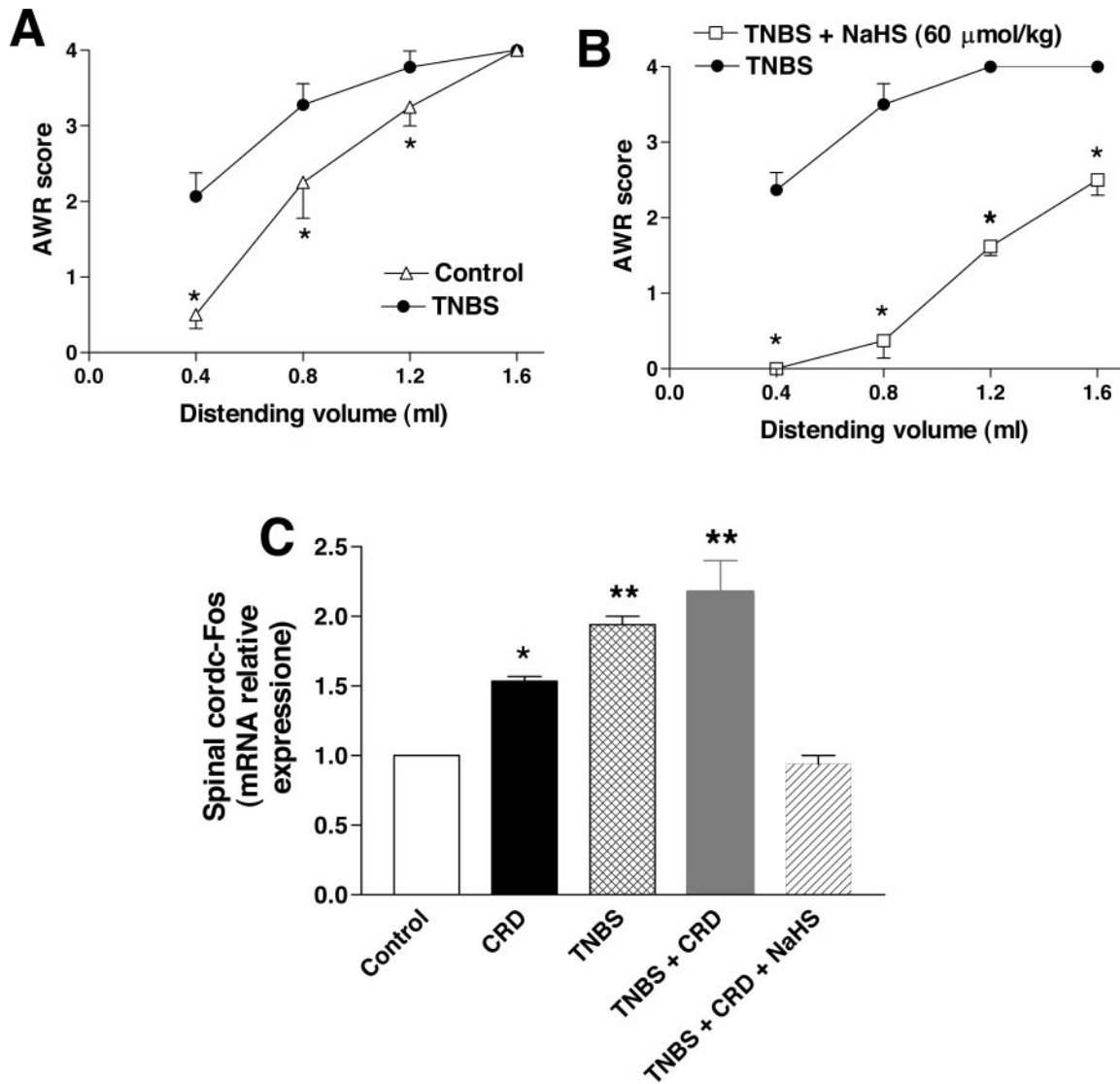


Fig. 7. H₂S maintains its antinociceptive effect in colitis. Colonic inflammation induced by TNBS causes allodynia and hyperalgesia (A) that are completely reversed by NaHS treatment (B). These results are confirmed by RT-PCR analysis of spinal c-Fos mRNA expression (C). Data are mean \pm S.E. of five rats. *, $p < 0.05$ versus control. **, $p < 0.05$ versus CRD alone.

served with doses of H₂S that fails to increase colon compliance. This finding is consistent with the observation that H₂S exerts a biphasic effect on colonic compliance. Indeed, in vitro experiments have demonstrated that NaHS at concentrations below 100 μ M induces a slight contraction of an isolated colonic segment, whereas concentrations up to 100 μ M are required to cause colon relaxation (E. Distrutti, unpublished data). A third more likely explanation for our results would be that the antinociceptive effect of H₂S is mediated by a direct inhibitory modulation of colorectal afferent pathways rather than the relaxation of colonic smooth muscle cells. Consistent with this view is the demonstration that H₂S-generating enzymes CSE and CBS are expressed in the spinal cord and colon and that detectable amounts of H₂S are produced by these tissues in presence of L-cysteine, a CSE/CBS substrate. Furthermore, the antinociceptive and relaxant actions of L-cysteine are inhibited by DL-propargylglycine, a CSE inhibitor, suggesting that generation of H₂S mediates the effect of L-cysteine. Finally, we found that H₂S administration decreased spinal cord expression of c-Fos mRNA.

Because induction of c-FOS expression is widely used as an index of nociception and activation of afferent pathways (Bonaz et al., 2000), our data support the notion that H₂S functions as a neuromodulator that participates in the inhibitory modulation of visceral nociception in the CNS.

In the present study, we have provided evidence that ATP-sensitive K⁺ channels mediate, at least in part, the antinociceptive activity of H₂S. Support for this concept comes from the observation that glibenclamide, a K_{ATP} channel blocker (Edwards and Weston, 1993), reverses the antinociceptive activity of H₂S, whereas pinacidil, an ATP-sensitive K⁺ channels opener, reproduces the same antinociceptive effect of NaHS on AWR response and abrogates spinal c-Fos mRNA expression induced by CRD (Zhao et al., 2001). The possibility that glibenclamide administration reverses the antinociceptive action of H₂S by simply inducing hyperalgesia and/or allodynia by itself is unlikely because, as previously shown by others (Ortiz et al., 2002), glibenclamide alone did not induce an hyperalgesic response to CRD.

Previous studies have shown that some actions of H₂S

might involve interactions with NO (Hosoki et al., 1997). In the cardiovascular system, the vasorelaxant properties of H₂S are greatly enhanced by NO (Hosoki et al., 1997). Moreover, NO has been implicated in the nociceptive neural pathways, acting both at the periphery (primary afferent neurons and dorsal root ganglia) and centrally in the brainstem and sensory structures of the thalamus (Mao, 1999), but its role in mediating visceral hyperalgesia and pain is still controversial. Although some data indicate that inhibition of NO synthesis exacerbates pain in models of visceral hyperalgesia (Zhuo et al., 1993), several studies have emphasized the pronociceptive role of this gaseous neurotransmitter (Malmberg and Yaksh, 1993; Minami et al., 1995). Here we have shown that L-NAME abrogates the H₂S-induced antinociception, suggesting that the integrity of the NO pathway is essential for the inhibitory effect of this gas.

One interesting finding of our study was the demonstration that L-NAME decreases plasma levels of H₂S. In vascular tissues, it has been suggested that NO increases the uptake of L-cysteine and the expression of CSE. Moreover, because CBS is a heme-containing protein (Meier et al., 2001) and heme-containing proteins are common targets of NO, the activity of CBS might be influenced by NO (Wang, 2002). Indeed, NO may regulate H₂S production. On the other hand, H₂S may decrease the expression of NOS and may modify K_{Ca}²⁺ channels to decrease their sensitivity to NO. Our data seem to confirm these observations, indicating that a strict interaction exists between H₂S and NO in the control of CRD-induced visceral nociception and H₂S production. The level(s) of the cross-talk between the two gaseous neuromodulators is unknown, but it is probable that NO acts by modulating both the H₂S production and effect of H₂S on visceral sensitivity and pain.

Recent studies have provided evidence that H₂S activates capsaicin-sensitive pathways in isolated bladder preparations (Patacchini et al., 2004). However, while in this system, the capsaicin-dependent effect of H₂S results in a vigorous concentration-dependent contractile response; our *in vivo* (and *in vitro*) findings indicate that NaHS increases colon compliance. Thus, the relevance of capsaicin-sensitive pathways to the effects of H₂S on colon nociception remains unclear.

The antinociceptive action of H₂S is maintained in a rodent model of postinflammatory pain. In animal models of acute (Ness and Gebhart, 1990) and chronic (Julia et al., 1995) inflammation, abnormal pain responses to CRD have been observed, demonstrating that inflammation induces both hyperalgesia and allodynia that persisted also when local inflammation is partially or totally resolved. Human studies in patients with ulcerative colitis and Crohn disease (Bernstein et al., 1996; Chang et al., 2000) and IBS (Collins et al., 2001) have confirmed these experimental findings. Many inflammatory and noninflammatory agents are thought to be involved in acute and chronic phases of intestinal inflammation and in the subsequent induction of hyperalgesia and/or allodynia. In the present study, the AWR score markedly increased during the lower levels (0.4 and 0.8 ml of water) of CRD in colitic rats, confirming that TNBS-induced inflammation determines allodynia. Moreover, c-Fos mRNA expression increased in rats with colitis in comparison with healthy controls, suggesting that colonic inflammations activates a

population of second order spinal cord neurons (Traub et al., 1992). One interesting observation of our study was the demonstration that H₂S administration completely reversed the allodynic effect of TNBS-induced colitis and markedly reduced pain related to the maximal CRD. The fact that the antinociceptive action of H₂S was associated with inhibition of c-FOS mRNA expression is therefore consistent with the notion that this gas acts as a direct neuromodulator of the afferent, sensitive spinal fibers. In contrast, the possibility that H₂S acts directly as an anti-inflammatory agent in this model is unlikely. Indeed, although we have shown that H₂S protects the gastric mucosa in rats administered anti-inflammatory drugs (Fiorucci et al., 2005), H₂S acts as a proinflammatory mediator. Thus, not only the *i.p.* administration of sodium hydrosulfide to mice increases lung and liver MPO activity and plasma levels of tumor necrosis factor α (Li et al., 2005) but treatment with DL-propargylglycine, an inhibitor of CSE, significantly attenuates carrageenan-induced hindpaw edema in a dose-dependent manner (Bhatia et al., 2005). In addition, any anti-inflammatory effect of the H₂S can be excluded, because we administered NaHS two weeks after TNBS when the acute colitis was largely resolved.

In summary, we have shown that systemic administration of NaHS increases the tolerance of rats to colorectal distension, irrespective of whether the mucosa is normal or inflamed. Although NaHS has antinociceptive effects that are independent of effects on smooth muscle contractility, it cannot be excluded the importance of the smooth muscle effects to the overall pharmacology of drug, particularly at higher doses. The presumed neurophysiological basis for these actions involves the activation of K_{ATP} channels and NO. Whether H₂S-releasing drugs may have utility in the treatment of painful functional and organic intestinal diseases remains to be investigated.

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