Evidence that hydrogen sulfide exerts antinociceptive effects in the gastrointestinal tract by activating K_{ATP} channels

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Abbreviations

H_2S, hydrogen sulphide; CBS, cystathionine β-synthase; CSE, cystathionine-γ-liase; CRD, colorectal distension; MPO, myeloperoxidase; NO, Nitric oxide; NaHS, sodium hydrogen sulfide.

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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-liase (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 water) colorectal distensions (CRDs) were produced in conscious rats (healthy and post-colitic) and rectal nociception assessed by measuring the behavioural response during CRD. Healthy rats were administered with 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 μmol/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_{ATP} channel inhibitor, and N_{ω}-Nitro-L-arginine methyl ester hydrochloride, 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 post-colitic rats. This effect is mediated by K_{ATP} channels and NO. H_2S-releasing drugs might be beneficial in treating painful intestinal disorders.

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Introduction

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). While 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 pyrodoxal-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-mercapto-sulphurtransferase) 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 (Wang, 2002; Zhao et al., 2001), while 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, 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 pinpoint CBS to be the major H_2S producing enzyme in neural tissues (Abe and Kimura, 1996).

H_2S regulates key neuronal functions, including the induction of ippocampal long-term potentiation, a synaptic model of learning and memory (Abe and Kimura, 1996; Kimura, 2000) and the release of the corticotropin releasing hormone from the hypothalamus (Russo et al., 2000). While the molecular mechanisms involved in these activities are only

partially known, it has been shown that H_2S 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 H_2S causes a cAMP-dependent potentiation of N-Methyl-D-Aspartate (NMDA) receptors (Moore et al, 2003). Previous studies have shown that, at low concentrations, H_2S enhances the smooth muscle relaxation effect of NO, suggesting that a "cross talk" between the two gases exists (Hosoki, 1997). Further, 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 H_2S modulates visceral nociception during CRD is still unknown.

In this study we have investigated the effects of H_2S administration in rodent models of visceral nociception. Our results demonstrate that H_2S modulates nociception induced by CRD in healthy and colitic rats, providing the ground for development of H_2S based therapy for treatment of painful abdominal condition in humans.

Methods

Materials

Sodium hydrogen sulfide (NaHS), L-cysteine, glibenclamide, pinacidil, N_{ω}-Nitro-L-arginine methyl ester hydrochloride (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 (S. Louis, MO, USA). The stock solution of NaHS was freshly prepared by dissolving NaHS immediately before use. Tissue Protein Extraction Reagent (T-PER) was obtained by Pierce Biotechnology (Rockford, IL, USA). All the chemicals were of analytical grade and were used without treatment. Deionized water filtered was used for the buffer preparation. Silver and sulfide ion selective electrode was from ThermoOrion (Beverly, MA, USA).

Animals

Male Wistar rats (200-250 g, Charles River, Monza, Italy) were housed in plastic cages and maintained under controlled conditions with 12-hours light/dark cycles with lights on at 7.00 AM. Tap water and standard laboratory chow were freely available. Food was withheld for 12 hours before surgical procedures and CRD recordings. After recovery from surgery, the rats were individually trained by spending 2-3 hours per day in a Plexiglas cage for 2-3 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 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 anaesthetized with pentobarbital (60 mg/kg intraperitoneally [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 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 to continuously monitoring the colorectal pressure by a computer (PowerLab PC, A.D. Instruments, Milford, MA, USA) and to a syringe for inflation/deflation of the balloon. The rats were then housed in a small Plexiglas cage (20 x 8 x 8 cm) on an elevated platform and allowed to regain consciousness and adapt for 1 hour. After recovery from sedation, the rats underwent the CRD procedure and behavioral response was tested in all groups except control group in which no CRD was performed. CRD of 20 seconds performed every 5 minutes was applied in increment of 0.4 ml starting from 0.4 ml and increasing to 1.6 ml 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 a double sets of CRD. Ten

minutes after the first CRD (0.4-1.6 ml water), drugs were administered intraperitoneally (i.p.) and/or intravenously (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 the 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 (LS) and assignment of an AWR score according with the behavioral scale previously described (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 sever 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 (about 20%). To determine the effect of H_2S on colonic smooth muscle, the compliance of the colon during CRD was obtained from colorectal volume and pressure and expressed as ml/mmHg.

Effects of H_2S on colonic nociception

The control group (n=5) consisted of fasting rats that underwent to surgical procedures but not CRD. To investigate whether H_2S administration modulates sensitivity and pain

induced by CRD, rats were treated i.p. with NaHS (as H_2S donor) at doses of 15, 30 o 60 µmol/kg (NaHS group), L-cysteine (the natural substrate for H_2S 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 H_2S donor for the following reasons: 1) NaHS dissociates to Na^+ and HS^- in solution, then HS^- associates with H^+ and produces H_2S. At physiological pH, ≈ one-third of the H_2S exists as the undissociated form (H_2S) while the remaining two-thirds is HS^- at equilibrium with H_2S (Beauchamp et al., 1984); 2) the use of NaHS enables us to define the concentrations of H_2S in solution more accurately and reproducibly then bubbling H_2S gas; 3) the influence of Na^+ ions (less than 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 H_2S.

The involvement of K_{ATP} channels in the modulation of visceral perception by H_2S was assessed by pre-treating rats with glibenclamide (K_{ATP} channels blocker) at a dose of 2.8 µmol/kg i.v. for 20 minutes before NaHS (60 µmol/kg i.p.) administration (glibenclamide + NaHS group) or glibenclamide alone (glibenclamide group). To confirm that the effects of H_2S on visceral perception is 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 administrated for 20 minutes between the two CRD sets (pinacidil group). To investigate whether NO is involved in the H_2S-mediated effects on visceral nociception, L-NAME, a non selective NO synthase inhibitor, was infused i.v. at the dose of 100 µmol/kg for 20 minutes 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 previously described (Fiorucci et al., 2002). Briefly, rats (14 animals) were anaesthetized 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 still alive underwent to CRD study as described above. In the first group we performed two consecutive series of CRD (TNBS + CRD group), while 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 previously described (Santucci et al., 1995).

Measurement of plasma H_2S concentration and H_2S production

Plasma H_2S concentrations and enzymatic capacity for H_2S production in colon and spinal cord were measured as described previously (Ubuka, 2002; Hosoki et al., 1997; Zhao et al., 2001) with modifications. Briefly, 250 µl of plasma were added to ice-cold 250 µl of NaOH 0.5 N in a sealed 3 neck reactor. A constant stream of nitrogen was passed through the mixture *via* gas-inlet capillary. The reactor was maintained at 37°C and H_2S 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 anti-oxidant buffer (SAOB) solution, consisting of 2 M KOH, 1 M salicylic acid and 0.22 M ascorbic acid at pH 12.8. After 30 minutes the SAOB solution was removed, and the sulfide concentration was measured with a sulfide sensitive electrode (Model 9616 S^{2-}/Ag^{+} electrode, Orion Research, Beverly, MA, USA) and expressed as H_2S (Ubuka, 2002; Khan et al., 1980).

One hundred mg of colon or spinal cord samples were homogenized in 1 ml of ice-cold T-PER protein extractor. The enzymatic capacity for H_2S production was performed on the same reactor as for the plasma analysis. Two ml 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. Reactions 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 minutes, 1 ml of 50% trichloroacetic acid solution was added to mixture to stop the reaction. The remainder H_2S in the mixture was carried out *via* nitrogen stream by other 30 minutes 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 manufacture specifications (Invitrogen, Milan, Italy). RNA was processed directly to cDNA by reverse transcription with Superscript II (Invitrogen). Briefly, 2 µg RNA was added to mixture which contain DNase I reaction buffer 10X and 1U DNase I. The mix was incubated 15 min at room temperature; than 4 µl of first strand buffer 5X (250 mM

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Tris-HCl pH=8.3; 375 mM KCl; 15 mM $MgCl_2$), 2 µl of DDT 0.1 M, 2 µl of dNTP's mix 10 mM, 1 µl of random primers 300 ng/µl, 0.5 µl of RNase out and 0.5 µl of Super Script II were added to the sample. 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 CBS the sense primer was: CCAGGACTTGGAGGTACAGC and the antisense TCGGCACTGTGTGGTAATGT; for rat CSE the sense primer was: GTATTGAGGCACCAACAGGT and the antisense GTTGGGTTTGTGGGTGTTTC; for the rat c-Fos the sense primer was: GTCTGGTTCCTTCTATGCAG and the antisense AGGTAGTGCAGCTGGGAGT. In control experiments with 3 replicates, no false positive were detected. Amplification reactions contained 2 µl cDNA, 12.5 µl of the 2X 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 (Biorad, Hercules, CA) and thermal cycling conditions were: 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.

Statistical analysis

All data are presented as the mean ± SEM, with sample sizes of at least 5 rats/group; statistical comparisons of unpaired data was performed by the Mann-Whitney test, while statistical comparisons of paired data was performed by the Wilcoxon signed rank test. An associated probability (p value) of less that 5% was considered significant.

Results

CBS and CSE are expressed in the colonic tissue and spinal cord

As illustrated in Figure 1A, mRNAs encoding for CBS and CSE, the two key enzymes involved in H_2S 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 H_2S generation (Figure 1B, left side). In the presence of L-cysteine the colon generated ≈ 2 nmol/min/g protein of gaseous H_2S, while spinal cord homogenates incubated at the same conditions produced ≈ 6 nmol/min/g protein of gaseous H_2S. Plasma H_2S concentration was ≈ 50 μM, similar to that reported previously by others (Guidotti 1996). The amounts of H_2S detected in the rat colon and spinal cord did not change during CRD alone or after NaHS or L-cysteine administration (data not shown).

H_2S 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 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 water) elicited volume dependent increases in the AWR score which were rapid in onset and persisted for the duration of the distension period (Figure 2A) with no significant reduction in colorectal pressure (Figure 2B). Distensions with 0.4 ml water induced a slight increment of the AWR score (less than 1) that was associated with a small rise of colorectal pressure (≈ 20 mmHg), indicating that this CRD represents a non-painful stimulus, while distensions with 1.2 and 1.6 ml water

were related to the maximal AWR scores (3 and 4 respectively) and to a very high colorectal pressures (up to 80 mmHg), 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 µmol/kg) there was no effect on AWR score (Figure 2C) or colorectal compliance (Figure 2D), while at doses of 30 and 60 µmol/kg, NaHS significantly reduced the CRD-induced AWR at all volumes of distension applied (Figures 2E and 2G, respectively). The decrease in the AWR response was associated to a decrease in rectal compliance only with the higher dose of NaHS (Figure 2H), suggesting that at the lower doses (15-30 µmol/kg) NaHS failed to induce colonic smooth muscle relaxation (Figure 2D and 2F, respectively).

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

Data on the antinociceptive effects of H_2S during CRD were confirmed by analysis of c-Fos expression in the spinal cord. Quantitative RT-PCR of cFOS mRNA expression demonstrates that CRD induced a two-fold increase in spinal c-Fos expression (n=5; $P<0.05$ versus control), confirming its nociceptive action (Figure 4). Administration with either NaHS or L-cysteine abrogated cFOS mRNA induction caused by CRD, indicating that the reduced AWR score was due to the antinociceptive effect of H_2S rather than a possible effect of this gas on the consciousness state (n=5; $P<0.05$ versus CRD). Basal plasma concentrations of H_2S were ≈ 47 µM and did not change during CRD, or following treatment with NaHS or L-cysteine (data not shown).

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K_{ATP} channels inhibition reverts antinociceptive effect of H_2S

To determine whether or not ATP-sensitive K^+ channels were involved in the antinociceptive effect of H_2S, the interaction of H_2S with known K_{ATP} channel modulators was examined. The inhibitory effect of NaHS on CRD-induced pain was completely reversed by pre-treating rats with 2.8 µmol/kg glibenclamide, a K_{ATP} channel antagonist, (Figure 5A). Moreover, glibenclamide inhibited colonic smooth muscle relaxation-induced by the highest dose of NaHS (Figure 5B). The antinociceptive effect of NaHS was mimicked by the K_{ATP} channel opener pinacidil (Figure 5C). Similarly to NaHS, pinacidil administration increased colonic compliance (Figure 5D). In contrast, treating rats with glibenclamide alone had no effect on CRD-induced nociception or colonic compliance (Figures 5E and 5F, respectively). Analysis of c-Fos expression confirmed the colorectal nociception and pain data. As illustrated in Figure 5G, pre-treatment with glibenclamide had no effect, while pinacidil respective down-regulated the c-Fos mRNA expression induced by CRD. Plasma H_2S concentrations did not significantly change during K_{ATP} channel modulators treatments (data not shown).

NO synthase inhibition reverts antinociceptive effect of H_2S

Pre-treating rats with L-NAME at the dose of 100 µmol/kg i.v. 10 minutes before NaHS administration (60 µmol/kg i.p.) produced an almost complete inhibition of the antinociceptive (Figure 6A) and relaxant (Figure 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 pre-treatment (Figure 6C). L-NAME administration reduced the plasma concentrations of H_2S when compared with those of control animals and other treatments (Figure 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).

H_2S inhibits pain in inflamed rats

The rats with colitis exhibited a loss of weight of about 20% when compared with healthy rats, and diarrhea was observed during the first week after induction of colitis. Macroscopic inflammation and thickening of bowel wall was observed in TNBS-treated rats compared with controls, while 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 2 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 Figure 7A, an increased nociception was observed during the low volume distensions (0.4 and 0.8 ml water), indicating that colonic inflammation induces allodynia (perception of non painful stimulus as painful) and hyperalgesia (perception of painful stimulus as more painful) to CRD. The AWR score determined during repeated CRD did not change, while pre-treating colitic rats with NaHS (60 µmol/kg i.p.) almost completely inhibited the allodynic response to CRD (Figure 7B). These data were confirmed by the analysis of spinal c-Fos mRNA: the expression of c-Fos mRNA in the spinal cord was 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 cFOS mRNA expression to values similar to that of controls (Figure 7C).

Discussion

The studies described herein demonstrate that H_2S 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 H_2S 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, while 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 H_2S. *First*, H_2S 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, H_2S may blunt sensorial functions causing a loss of consciousness that mimics a pain-free condition during CRD. However this is an unlikely explanation since we did not observe any change in the consciousness during these studies. In addition, endogenously generated H_2S is rarely accumulated or toxic to cells due to the cellular metabolism of the gas. Finally, concentrations <30 µM H_2S cause no apparent disturbance in oxidative phosphorylation due to the rapid oxidation of H_2S in mithocondria (Guidotti, 1996). Recent data demonstrated that physiologic plasma H_2S concentrations in rats are ≈ 46 µM (Calderone at al., 1996). In the present study the plasma concentrations of H_2S ranged from 30 to 55 µM, making unlikely a central toxic effect of the gas. A *second* explanation is that H_2S 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 H_2S significantly inhibited CRD-induced nociception at the doses of 30 and 60 µmol/kg, but increased rectal compliance only at the higher dose. Thus, while H_2S 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 observed with doses of H_2S that fails to increase colon compliance. This finding is consistent with the observation that H_2S exerts a byphasic effect on colonic compliance. Indeed, *in vitro* experiments have demonstrated that NaHS at concentrations below 100 µM induces a slight contraction of an isolated colonic segments, while concentrations up to 100 µM are required to cause colon relaxation (Distrutti et al., unpublished). A *third*, more likely explanation for our results would be that the antinociceptive effect of H_2S is mediated by a direct inhibitory modulation of colorectal afferent pathways rather than to the relaxation of colonic smooth muscle cells. Consistent with this view is the demonstration that H_2S generating enzymes, CSE and CBS, are expressed in the spinal cord and colon and that detectable amounts of H_2S 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 H_2S mediates the effect of L-cysteine. Finally, we found that H_2S administration decreased spinal cord expression of c-Fos mRNA. Since induction of cFOS expression is widely used as an index of nociception and activation of afferent pathways (Bonaz et al., 2000), our data support the notion that H_2S 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 H_2S. Support for this concept

comes from the observation that glibenclamide, a K_{ATP} channel blocker (Edwards and Weston, 1993), reverses the antinociceptive activity of H_2S, while 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 H_2S by simply inducing hyperalgesia and/or allodynia by itself is unlike since, 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 H_2S might involve interactions with NO (Hosoki, 1997). In the cardiovascular system, the vasorelaxant properties of H_2S are greatly enhanced by NO (Hosoki, 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 pro-nociceptive role of this gaseous neurotransmitter (Malmberg and Yaksh, 1993; Minami et al., 1995). Here we have shown that L-NAME abrogates the H_2S-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 H_2S. In vascular tissues, it has been suggested that NO increases the uptake of L-cysteine and the expression of CSE. Moreover, since 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 H_2S production. On the other hand, H_2S may decrease the expression of NOS

and may modify K_{Ca2+} channels to decrease their sensitivity to NO. Our data seem to confirm these observations indicating that a strict interaction exists between H_2S and NO in the control of CRD-induced visceral nociception and H_2S production. The level(s) of the cross-talk between the two gaseous neuromodulators is unknown but is probable that NO acts by modulating both the H_2S production and effect of H_2S on visceral sensitivity and pain.

Recent studies have provided evidence that H_2S activates capsaicin-sensitive pathways in isolated bladder preparations (Patacchini et al., 2004). However, while in this system the capsaicin-dependent effect of H_2S 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 H_2S on colon nociception remains unclear.

The antinociceptive action of H_2S is maintained in a rodent model of post-inflammatory 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 at al., 1996; Chang at al., 2000) and IBS (Collins et al., 2001) have confirmed these experimental findings. Many inflammatory and non inflammatory 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 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 H_2S 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 H_2S was associated with inhibition of cFOS 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 H_2S acts directly as an anti-inflammatory agent in this model is unlikely. Indeed, while we have shown that H_2S protects the gastric mucosa in rats administered anti-inflammatory drugs (Fiorucci et al., 2005), H_2S 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 TNFα (Li et al., 2005) but, treatment with DL-propargylglycine, an inhibitors of CSE, significantly attenuates carrageenan-induced hindpaw oedema in a dose-dependent manner (Bhatia et al., 2005). In addition, any anti-inflammatory effect of the H_2S can be excluded, since 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 anti-nociceptive 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 H_2S-releasing drugs may have utility in the treatment of painful functional and organic intestinal diseases remains to be investigated.

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Legends to figures

Figure 1

CBS and CSE are expressed in the colon and spinal cord. As shown in panel A, RT-PCR expression of CSE and CBS in the colon (above) and spinal cord (below). The RT-PCR shown is representative of four. Endogenous production of H_2S by colon and spinal cord homogenates and physiological plasmatic concentrations of H_2S (panel B).

Figure 2

H_2S reverses CRD-induced nociception in conscious rats. CRD induces a volume-dependent, reproducible increment of the AWR (panel A) without a significant change of colorectal compliance (panel B). NaHS administration determines a dose-dependent inhibition of the nociceptive action of the CRD that is significant at the doses of 30 µmol/kg (panel E) and 60 µmol/kg (panel G), while the dose of 15 µmol/kg is ineffective (panel C). At the doses of 15 µmol/kg (panel D) and 30 µmol/kg (panel F) no change of the colorectal compliance is observed, while at the higher dose NaHS induces a significant colorectal relaxation (panel H). Data are mean ± SE of 5 rats. *P<0.05 versus control.

Figure 3

L-cysteine reproduces the effects of H_2S. L-cysteine causes a significant reduction of the AWR score (panel A) and relaxation of the colorectal muscular wall as demonstrated by the increment of the colorectal compliance (panel B). DL-propargylglycine, a CSE inhibitor, reverses antinociceptive (panel C) and relaxant (panel D) effects of L-cysteine. Data are mean ± SE of 5 rats. *P<0.05 versus control.

Figure 4

H_2S inhibits the expression of c-Fos mRNA in the spinal cord. CRD induces an over-expression of spinal c-Fos mRNA that is reversed by NaHS and L-cysteine administration. Data are mean ± SE of 5 rats. *P<0.05 versus control. ** P<0.05 vs CRD alone.

Figure 5

The antinociceptive effect of H_2S is modulated by K_{ATP} channels. Pre-treating rats with the K_{ATP} channel blocker glibenclamide abrogates the antinociceptive (panel A) and myorelaxant (panel B) effects of NaHS, while the K_{ATP} channel opener pinacidil reproduces the effects of NaHS on both the visceral sensitivity (panel C) and the colorectal compliance (panel D). Glibenclamide alone does not induce any changes of the AWR score (panel E) and the colorectal compliance (panel F). These results are confirmed by quantitative RT-PCR data on spinal c-Fos expression (panel G). Data are mean ± SE of 5 rats. *P<0.05 versus control. **P<0.05 vs CRD alone.

Figure 6

The antinociceptive effect of NaHS is modulated by NO. Pre-treating rats with the NOS inhibitor L-NAME abrogates the antinociceptive (panel A) and myorelaxant (panel B) effects of NaHS. Quantitative RT-PCR analysis of c-Fos mRNA expression in the spinal cord confirms the functional data (panel C). L-NAME administration reduces plasmatic H_2S concentration (panel D). Data are mean ± SE of 5 rats. *P<0.05 versus control.

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Figure 7

H_2S maintains its antinociceptive effect in colitis. Colonic inflammation induced by TNBS causes allodynia and hyperalgesia (panel A) that are completely reversed by NaHS treatment (panel B). These results are confirmed by RT-PCR analysis of spinal c-Fos mRNA expression (panel C). Data are mean ± SE of 5 rats. *P<0.05 versus control. **P<0.05 vs CRD alone.

Figure 1

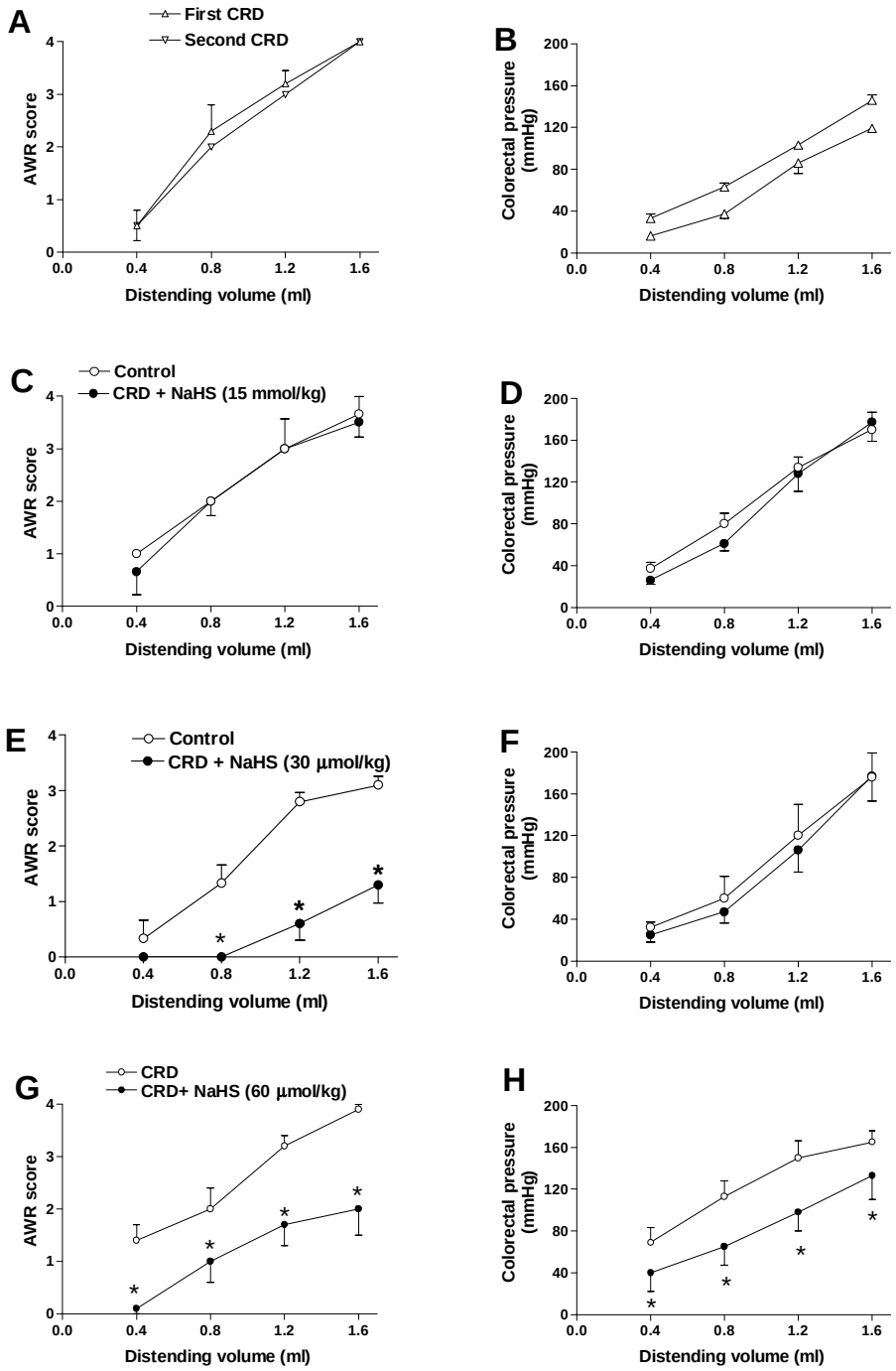

Figure 2

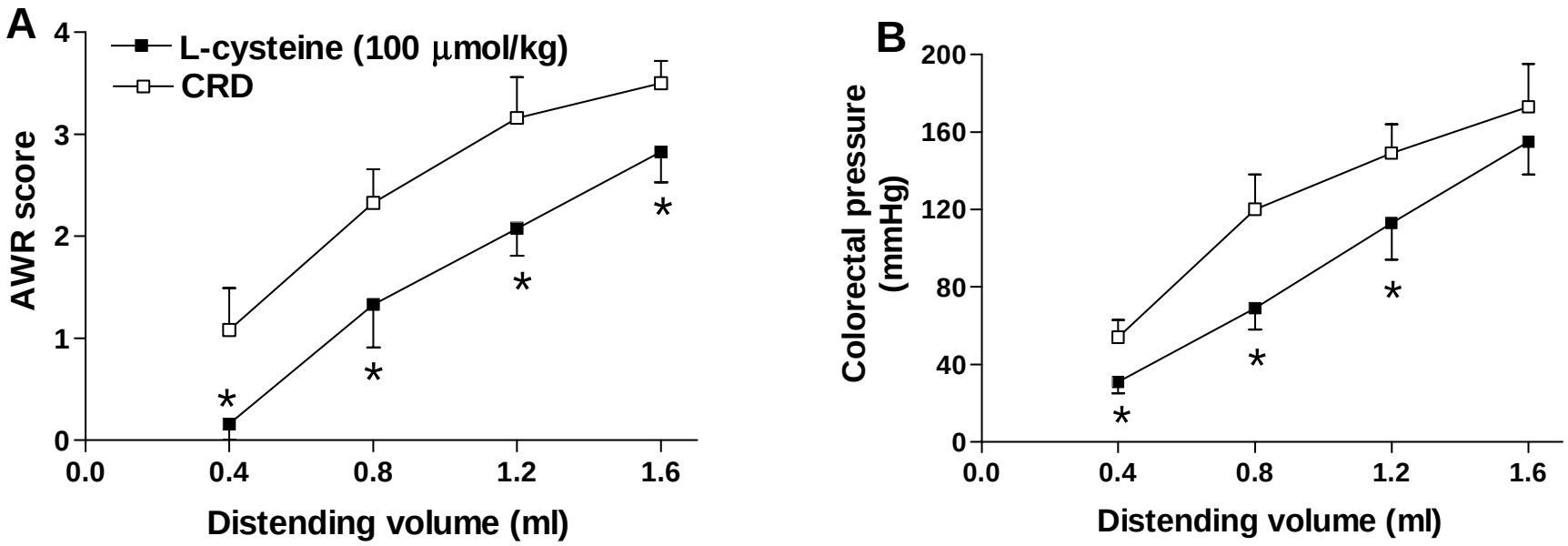

Figure 3

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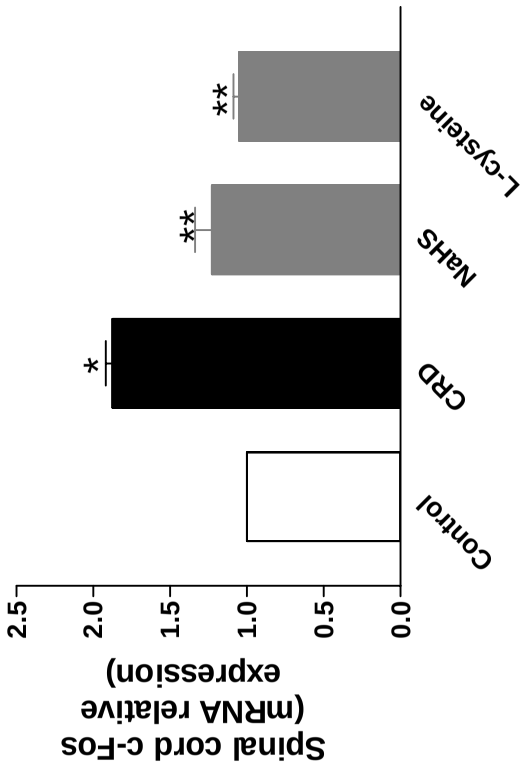

Figure 4

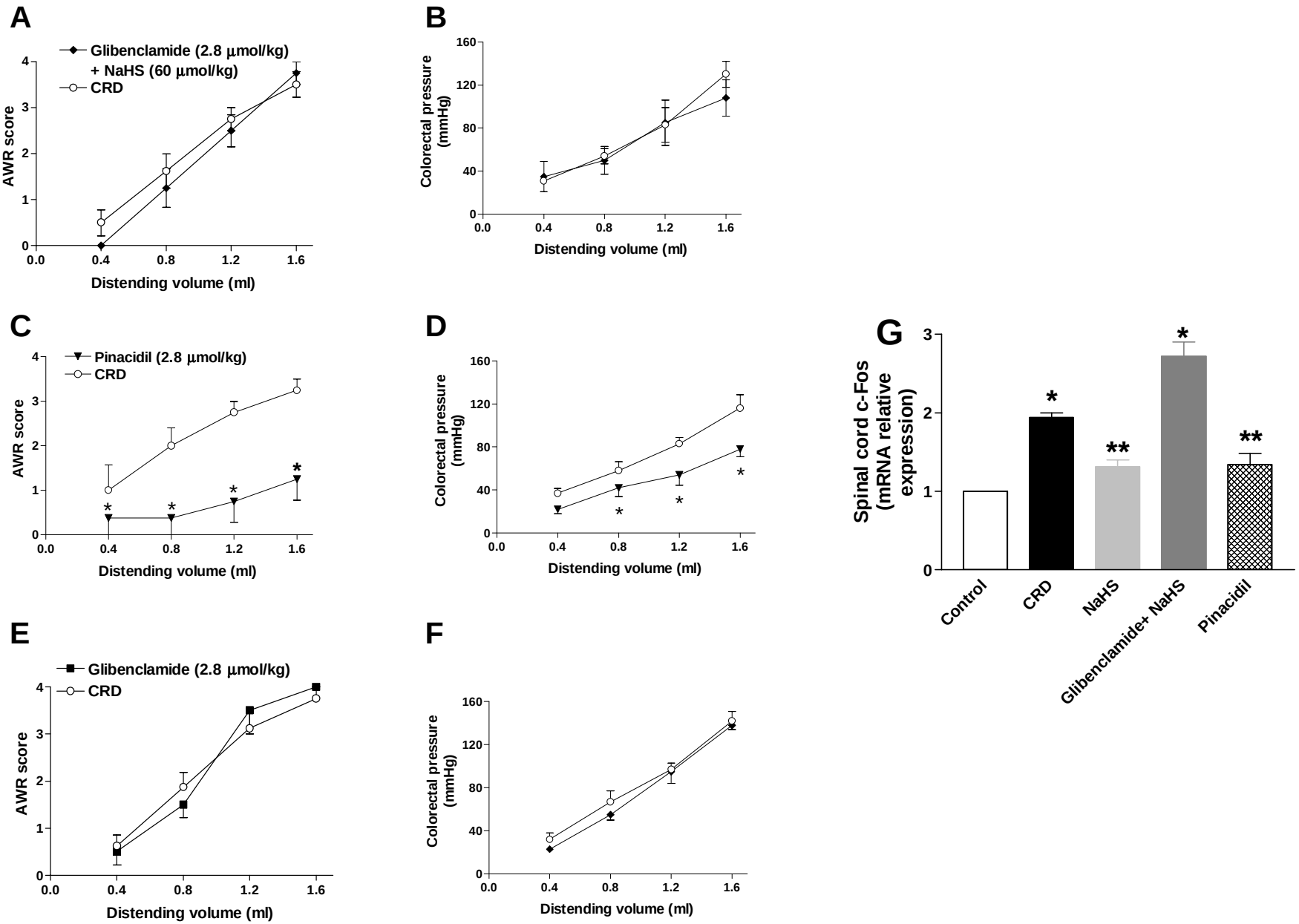

Figure 5

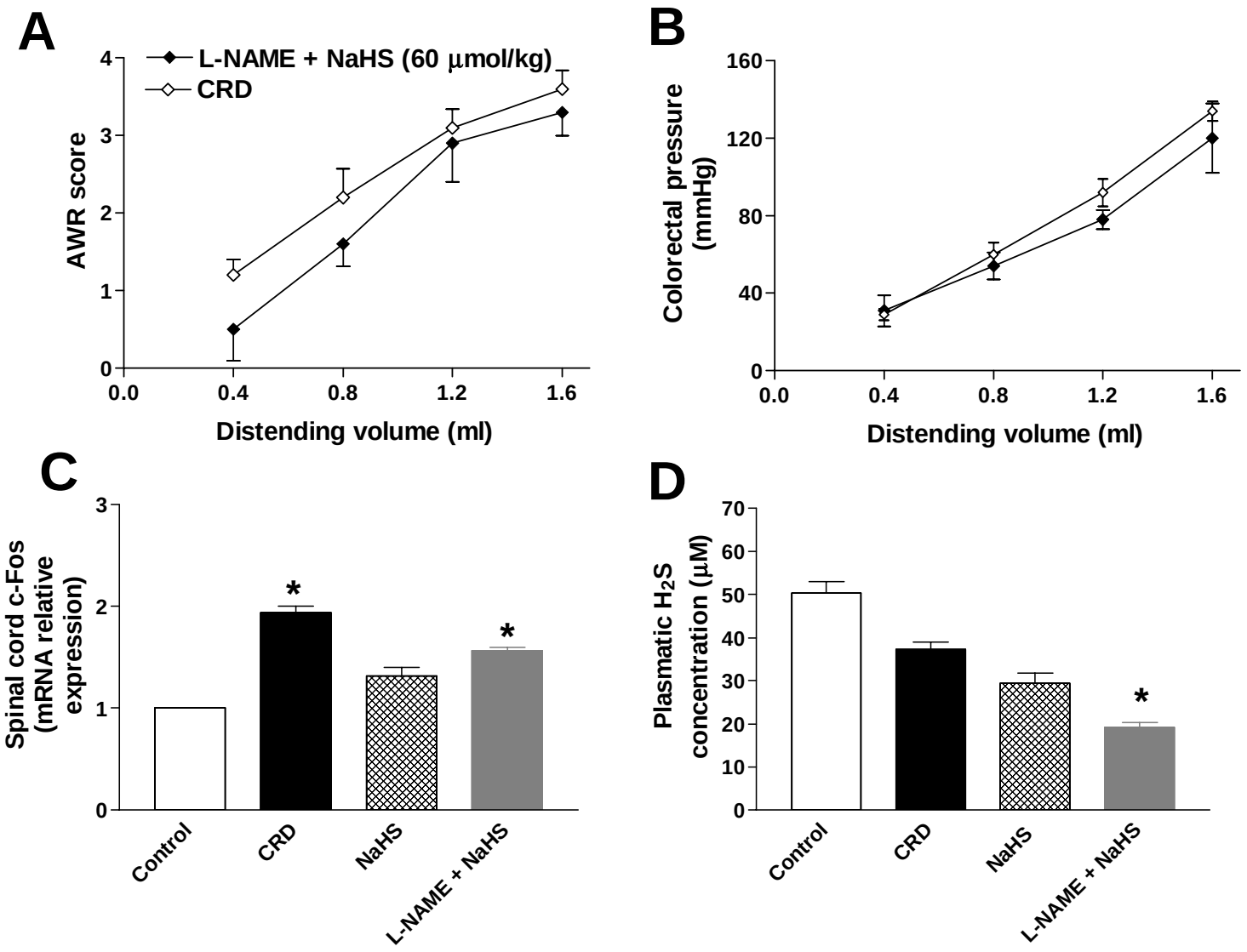

Figure 6

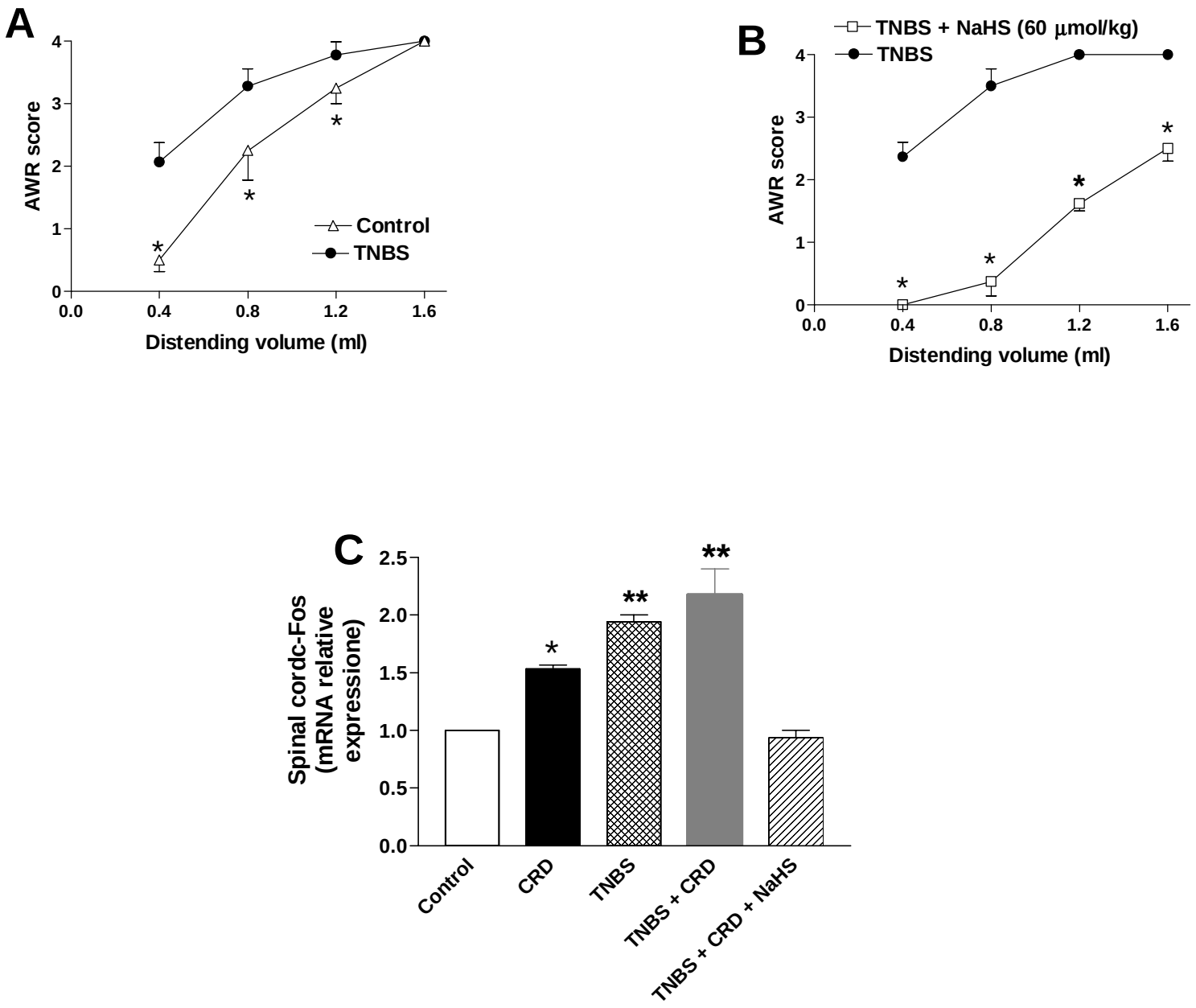

Figure 7