

## 5-Amino-2-hydroxybenzoic Acid 4-(5-Thioxo-5H-[1,2]dithiol-3yl)-phenyl Ester (ATB-429), a Hydrogen Sulfide-Releasing Derivative of Mesalamine, Exerts Antinociceptive Effects in a Model of Postinflammatory Hypersensitivity<sup>§</sup>

Eleonora Distrutti, Luca Sediari, Andrea Mencarelli, Barbara Renga, Stefano Orlandi, Giuseppe Russo, Giuseppe Caliendo, Vincenzo Santagada, Giuseppe Cirino, John L. Wallace, and Stefano Fiorucci

*Dipartimento di Medicina Clinica e Sperimentale, University of Perugia, Perugia, Italy (E.D., L.S., A.M., B.R., S.O., G.R., S.F.); Dipartimento di Chimica Farmaceutica e Tossicologia e Dipartimento di Farmacologia Sperimentale, University of Naples, Naples, Italy (G.Ca., V.S., G.Ci.); and Inflammation Research Network, Department of Pharmacology, University of Calgary, Calgary, Alberta, Canada (J.L.W.)*

Received April 18, 2006; accepted July 17, 2006

### ABSTRACT

H<sub>2</sub>S functions as a neuromodulator and exerts anti-inflammatory activities. Recent data indicate that irritable bowel syndrome (IBS) is linked to inflammation of the gastrointestinal tract. In this study, we have investigated the role of a novel H<sub>2</sub>S-releasing derivative of mesalamine (5-amino-2-hydroxybenzoic acid 4-(5-thioxo-5H-[1,2]dithiol-3yl)-phenyl ester, ATB-429) in modulating nociception to colorectal distension (CRD), a model that mimics some features of IBS, in healthy and postcolitic rats. Four graded (0.4–1.6 ml of water) CRDs were produced in conscious rats, and colorectal sensitivity and pain were assessed by measuring the abdominal withdrawal response and spinal c-Fos expression. In healthy rats, ATB-429 dose dependently (25, 50, or 100 mg/kg) attenuated CRD-induced hypersensitivity and significantly inhibited CRD-induced overexpression of spinal c-FOS mRNA, whereas mesalamine had no effect. ATB-429-induced antinociception was

reversed by glibenclamide, a ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channel inhibitor. The antinociceptive effect of ATB-429 was maintained in a rodent model of postinflammatory hypersensitivity (4 weeks after colitis induction). At a dose of 100 mg/kg, ATB-429 reversed the allodynic response caused by CRD in postcolitic rats. Colonic cyclooxygenase-2 and interleukin-1 $\beta$  mRNA and spinal c-FOS mRNA expression were significantly down-regulated by ATB-429, but not by mesalamine. ATB-429, but not mesalamine, increased blood concentrations of H<sub>2</sub>S in both healthy and postcolitic rats. Taken together, these data suggest that ATB-429 inhibits hypersensitivity induced by CRD in both healthy and postcolitic, allodynic rats by a K<sub>ATP</sub> channel-mediated mechanism. This study provides evidence that H<sub>2</sub>S-releasing drugs might have beneficial effects in the treatment of painful intestinal disorders.

Irritable bowel syndrome (IBS) is a common disorder involving the gastrointestinal tract usually defined by the co-

existence of abdominal pain or discomfort and an alteration in bowel habit that cannot be explained by a structural or biochemical abnormality (Thompson et al., 1999). Although the underlying pathophysiological mechanisms remain obscure, psychosocial disturbances, gastrointestinal dysmotility, and altered visceral perception are currently the most accepted hypothesis.

Animal models have provided evidence that inflammation-driven sensory afferent system activation might be a causative factor for development of altered visceral perception. Support for this view comes from the observation that allo-

J.L.W. is supported by grants from the Canadian Institutes of Health Research and holds a Canada Research Chair in Inflammation Research and an Alberta Heritage Foundation for Medical Research Scientist award. G.C., V.S., G.C., J.L.W., and S.F. hold shares in Antibe Therapeutics Inc., the manufacturer of ATB-429.

<sup>§</sup> The online version of this article (available at <http://jpet.aspetjournals.org>) contains supplemental material.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

doi:10.1124/jpet.106.106435.

**ABBREVIATIONS:** IBS, irritable bowel syndrome; MPO, myeloperoxidase; IBD, irritable bowel disease; ABT-429, 5-amino-2-hydroxybenzoic acid 4-(5-thioxo-5H-[1,2]dithiol-3yl)-phenyl ester; K<sub>ATP</sub>, ATP-sensitive K<sup>+</sup>; CBS, cystathionine- $\beta$  synthase; CSE, cystathionine- $\gamma$  lyase; CRD, colorectal distension; ADT-OH, 5-*p*-hydroxyphenyl-1,2-dithione-3-thione; AWR, abdominal withdrawal reflex; TNBS, trinitrobenzene sulfonic acid; SAOB, sulfide antioxidant buffer; RT-PCR, reverse transcription-polymerase chain reaction; COX, cyclooxygenase; TNF, tumor necrosis factor; IL, interleukin; cNOS, constitutive NO synthase; CGRP, calcitonin gene-related peptide; TAC, takikinin; i.p., intraperitoneal; i.v. intravenous.

dynia and/or hyperalgesia develops in animals after resolution of acute infective (Barbara et al., 1997) or chemically induced (Julia et al., 1995; Distrutti et al., 2006) colitis, and by the finding that maternal deprivation-induced visceral hyperalgesia, a model that involves no direct manipulation of the colon, is associated with increased myeloperoxidase (MPO) activity, a measure of colon inflammation (Coutinho et al., 2002). Likewise, low-grade inflammation is found in the colon of IBS patients and is increasingly regarded as a putative causative factor for symptoms development. Support for this concept comes from the following evidence: first, epidemiological studies have shown that infective gastroenteritis is a risk factor for development of IBS-like symptoms, and this association is now referred to as postinfective IBS (Garcia-Rodriguez and Ruigomez, 1999); and second, histological and ultrastructural studies have shown that an increased number of mast cells and T cells are found in the lamina propria of the ileum and colon of IBS patients in comparison with healthy subjects (Barbara et al., 2006). Moreover, there is evidence that, in these postinflammatory states, immune cells localize in proximity to nerve endings, raising the possibility that mediators released from immune cells might alter enteric nerve function and muscle contractility (Barbara et al., 2004). Together, these observations support the concept that IBS is, at least in some patients, the consequence of the failure of the mucosal immune system to attenuate inflammatory response after the clearance of an infectious agent. Evidence does exist that genetic factors may play a role in maintaining intestinal inflammation in specific subsets of IBS patients (Gwee et al., 1996). Finally, further support for a link between inflammation and altered perception is provided by the observation that patients with chronic inflammatory bowel diseases (IBDs) develop IBS-like symptoms and visceral hyperalgesia during the quiescent phases of their diseases (Isgar et al., 1983; Rao et al., 1987). In these patients, mesalamine (5-amino-2-hydroxybenzoic acid) is widely used to maintain remission, although it has no effect in preventing development of IBS-like symptoms.

Gaseous transmitters are a growing family of regulatory mediators involved in regulation of physiological and pathological functions in mammalian tissues (Wang, 2002; Moore et al., 2003). Although NO is the best characterized member of this family, it is increasingly recognized that CO and H<sub>2</sub>S also exert regulatory functions (Wang, 2002; Moore et al., 2003). H<sub>2</sub>S regulates key neuronal functions, including the induction of hippocampal long-term potentiation, a synaptic model of learning and memory (Abe and Kimura, 1996), and the release of the corticotrophin-releasing hormone from the hypothalamus (Russo et al., 2000). In addition there is evidence that H<sub>2</sub>S may act as a proinflammatory mediator (Bhatia et al., 2005; Li et al., 2005). Although the molecular mechanisms involved in these activities are only partially known, it has been shown that H<sub>2</sub>S increases cAMP levels in neuronal and glial cell lines and primary neuron cultures and hyperpolarizes dorsal raphe neurons (Moore et al., 2003), contributes to cardioprotection (Pan et al., 2006), and affects insulin secretion from an insulin-secreting cell line (Yang et al., 2005) by activating ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels.

We have recently shown that cystathionine-β synthase (CBS) and cystathionine-γ lyase (CSE), the two major enzymes involved in H<sub>2</sub>S generation, are constitutively expressed in colon and spinal cord and that measurable quantities

of H<sub>2</sub>S are produced by these tissues. Moreover, H<sub>2</sub>S administration inhibits colorectal distension (CRD)-induced pain in healthy and allodynic rats by acting on the K<sub>ATP</sub> channels (Distrutti et al., 2006). Following these initial results, we have examined the effects of ATB-429, a new chemical entity that combines an H<sub>2</sub>S-releasing moiety with mesalamine. Here, we have compared the effects of ATB-429 to those of mesalamine in terms of alleviating CRD-induced hypersensitivity in healthy and postcolitic rats.

## Materials and Methods

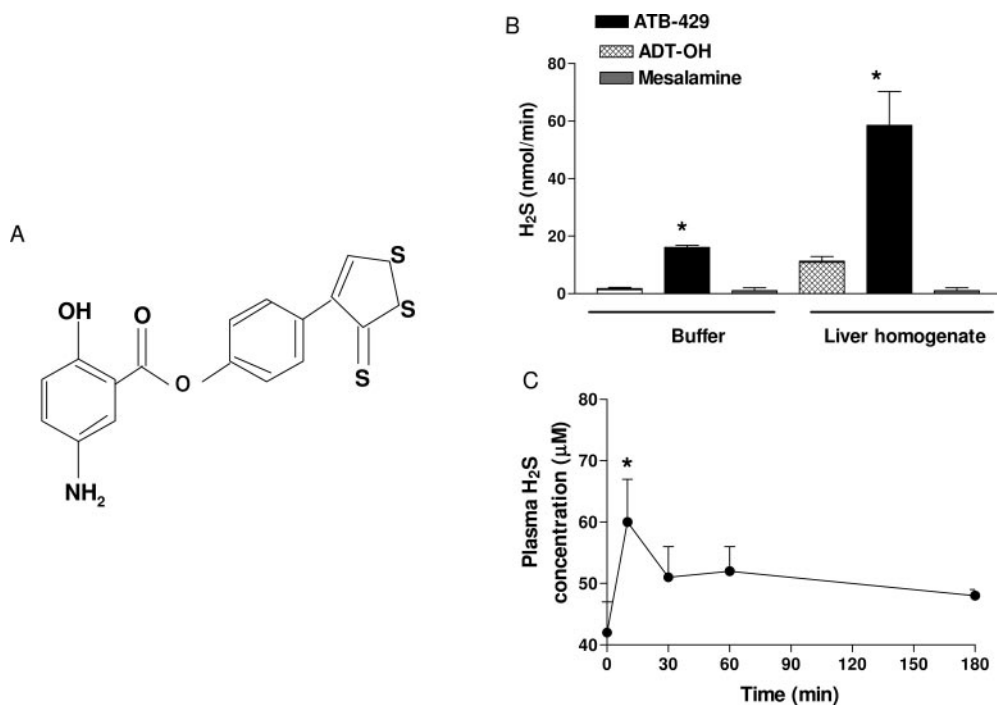
**Structure and Purity of ATB-429.** The chemical structure of 5-amino-2-hydroxy-benzoic acid 4-(5-thioxo-5*H*-[1,2]dithiol-3yl)-phenyl ester (ATB-429) is illustrated in Fig. 1, whereas key synthetic steps are shown in Supplemental Fig. 1. The structure of the ATB-429 was verified spectroscopically by proton <sup>1</sup>H NMR and <sup>13</sup>C NMR. Spectra were recorded on Varian Mercury Plus 400 instrument (Varian, Torino, Italy). Chemical shifts are referred to Me<sub>4</sub>Si as internal standard. Mass spectra of the synthesized products were performed on API 2000 mass spectrometry (Applied Biosystems International, Monza, Italy). Melting point was performed on Buchi B-540 instrument (BÜCHI Labortechnik AG, Flawil, Switzerland). Purity of the compound was 98%, as assessed by high-performance liquid chromatography (Varian, Torino, Italy). 5-*p*-Hydroxyphenyl-1,2-dithione-3-thione (ADT-OH) (Christen, 1995) was used as H<sub>2</sub>S-releasing moiety (Fig. 1A).

**Materials.** Ascorbic acid, salicylic acid, potassium hydroxide, L-cysteine, *N*-acetyl-L-cysteine, DL-propargylglycine, trichloroacetic acid, pyridoxal-5'-phosphate, polyethylene glycol, glibenclamide, mesalamine, and phosphate-buffered saline were purchased from Sigma-Aldrich (S. Louis, MO). ADT-OH and ATB-429 were provided by Antibe Therapeutics Inc. (Calgary, Alberta, Canada). Tissue protein extraction reagent T-PER was obtained by Pierce Chemical (Rockford, IL). All of 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).

**Animals.** Male Wistar rats (200–250 g; Charles River, Monza, 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, which allowed them to adjust to a movement-restricted 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 rats and were conducted in a blinded 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. After 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



**Fig. 1.** ATB-429 functions as an H<sub>2</sub>S donor. A, chemical structure of ATB-429 showing a mesalamine moiety linked to the H<sub>2</sub>S-releasing moiety (ADT-OH). B, ATB-429 releases H<sub>2</sub>S in vitro. Data are  $\pm$  S.E. of five experiments. \*,  $P < 0.05$ . C, time course of plasma H<sub>2</sub>S concentrations in rats administered ATB-429 (100 mg/kg i.p.). Groups of four to five rats were administered 100 mg/kg ATB-429 at time 0 and sacrificed at indicated time points. H<sub>2</sub>S concentrations in the blood were measured as described under *Materials and Methods*. Data are mean  $\pm$  S.E. \*,  $P < 0.05$  versus baseline.

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, ADInstruments, Milford, MA) and to a syringe for inflation/deflation of the balloon. The rats were then housed in a small Plexiglas cage (20  $\times$  8  $\times$  8 cm) on an elevated platform and 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 except 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. Animals underwent a double set of CRD. Ten minutes after the first CRD (0.4–1.6 ml of water), drugs were administered i.p. and/or intravenously (i.v.). Five minutes after the end of the drug 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 a 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 any behavioral response (i.e., score 0) were excluded from further study (approximately 20%). To determine the effect of H<sub>2</sub>S on colonic smooth muscle, the compliance of the colon during CRD was

obtained from colorectal volume and pressure and expressed as milliliters per mm Hg.

**Effects of ATB-429 on Colonic Hypersensitivity in Healthy Rats.** The control group ( $n = 5$ ) consisted of fasting rats that underwent the surgical procedures but not CRD, whereas the CRD group consisted of fasting, healthy animals that underwent surgical procedures and two sets of CRD. To investigate whether ATB-429 modulates sensitivity and pain induced by CRD, rats were treated i.p. with ATB-429 at doses of 25, 50, or 100 mg/kg (ATB-429 group), mesalamine at the dose of 100 mg/kg (mesalamine group), or vehicle (CRD group).

The involvement of K<sub>ATP</sub> channels in the modulation of visceral perception by H<sub>2</sub>S was assessed by pretreating rats with glibenclamide (K<sub>ATP</sub> channel blocker) at a dose of 2.8  $\mu$ mol/kg i.v. for 20 min before ATB-429 administration (glibenclamide plus ATB-429 group) or glibenclamide alone (glibenclamide group). At the end of the CRD procedures, rats were sacrificed and blood, colon, and spinal cord (L<sub>1</sub>–L<sub>5</sub>) were collected for further analysis.

**Induction of Colitis.** Colitis was induced as described previously (Wallace et al., 1989). In brief, rats were anesthetized with 60 mg/kg i.p. pentobarbital. Trinitrobenzene sulfonic acid (TNBS) at a 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 4 weeks, rats still alive were used for a CRD study, as described above. Five rats were immediately sacrificed and served as controls (TNBS group). In the other rats, we performed two consecutive series of CRD without administering drugs (TNBS plus CRD group;  $n = 5$ ), or we repeated CRD after treatment with ATB-429 at a dose of 100 mg/kg i.p. (TNBS plus CRD and ATB-429 group;  $n = 5$ ) or mesalamine at a dose of 100 mg/kg i.p. (TNBS plus CRD and mesalamine group;  $n = 5$ ). At the end of the CRD procedures, rats were sacrificed and blood, colon, and spinal cord were collected for further analysis.

**Assessment of Colonic Inflammation.** Colons were examined blindly 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 measurement of MPO activity, an index of granulocyte infiltration, as described previously (Santucci et al., 1995).

**In Vitro H<sub>2</sub>S Release.** To compare the in vitro H<sub>2</sub>S release induced by mesalamine, ATB-429, and ADT-OH, the H<sub>2</sub>S-releasing moiety of ATB-429, 100 to 150 mg of isolated livers was homogenized in 1 ml of ice-cold T-PER protein extractor. The H<sub>2</sub>S release was lead on the same reactor of plasma analysis. Two milliliters of an assay reaction mixture was introduced in the reactor. The mixture contained 10 mM ATB-429 or 10 mM ADT-OH dissolved in polyethylene glycol and 100 mM potassium phosphate buffer, pH 7.4. Incubations were lead with or without presence of 10% (w/v) liver homogenate and 2 mM pyridoxal 5'-phosphate. 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 into the second reactor containing 2 ml of sulfide antioxidant buffer (SAOB) as described previously. 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 H<sub>2</sub>S in the mixture was carried out via nitrogen stream by another 30 min of incubation at 37°C. The concentration of sulfide in the SAOB solution was measured with a sulfide-sensitive electrode as described previously (Khan et al., 1980; Ubuka, 2002).

**Measurement of Plasma H<sub>2</sub>S Concentrations.** To determine the kinetics of H<sub>2</sub>S released from ATB-429, groups of four to five rats were treated with ATB-429 at the dose of 100 mg/kg i.p. and sacrificed after 10, 30, 60, and 180 min. A time-course curve of plasma H<sub>2</sub>S concentrations was then constructed. Plasma H<sub>2</sub>S concentrations were measured as described previously (Zhao et al., 2001; Ubuka, 2002) with modifications. In brief, 250  $\mu$ l of plasma was added to ice-cold 250  $\mu$ l of 0.1 N NaOH in a sealed three-neck reactor. A constant stream of nitrogen was passed through the mixture via a gas inlet capillary. The reactor was maintained at 37°C, and H<sub>2</sub>S 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 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 S<sup>2-</sup>/Ag<sup>+</sup> electrode; ThermoOrion) and expressed as H<sub>2</sub>S (Khan et al., 1980; Ubuka, 2002).

**Reverse Transcription-Polymerase Chain Reaction on Colonic and Spinal Tissues.** Whether acute administration of ATB-429 could modulate the expression of genes that participate in the control of inflammation and pain was studied by determining the colonic and spinal cord expression of mRNA of CBS, CSE, c-Fos, cyclooxygenase (COX)-1 and -2, tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , constitutive NO synthase (cNOS), calcitonin gene-related peptide (CGRP), and takikinin (TAC)-1 and -2 in postcolitic rats. In brief, total RNA was isolated from rat colon and spinal cord by using the TRIzol reagent according to manufacturer's specifications (Invitrogen, Milan, Italy). RNA was processed directly to cDNA by reverse transcription with Superscript II (Invitrogen). Two micrograms of RNA was added to a mixture of 10 $\times$  DNase I reaction buffer and 1 U of DNase I. The mix was incubated 15 min at room temperature, and then 4  $\mu$ l of 5 $\times$  first strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, and 15 mM MgCl<sub>2</sub>), 2  $\mu$ l of 0.1 M dithiothreitol, 2  $\mu$ l of 10 mM dNTPs mixture, 1  $\mu$ l of random primers at 300 ng/ $\mu$ l, 0.5  $\mu$ l of RNase Out, and 0.5  $\mu$ l 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 polymerase chain reaction primers for quantitative and qualitative polymerase chain reaction were synthesized by MWG Biotech (High Point, NC) and designed using software PRIMER3-NEW using published sequence data from the National Center for Biotechnology Information database. Table 1 illustrates the rat primers (sense and antisense) used in this study. In control experiments with three replicates, no false positives were detected. Amplification reactions contained 2  $\mu$ l of cDNA, 12.5  $\mu$ l of the 2 $\times$  dynamo SYBR Green qPCR Master Mix, and 0.75  $\mu$ l of each of the

TABLE 1

Rat primers (sense and antisense) used in this study

Gene	Sense Primer (5'-3')	Antisense Primer (5'-3')
<i>rCBS</i>	ccaggacttgaggtacagc	tggcactgtgtggtaatgt
<i>rCSE</i>	gtattgaggcaccacaggt	gttgggtttgtgggtgttc
<i>rcFOS</i>	gtctgttctctctatcgag	taggtagtgcagctgggagt
<i>rCOX1</i>	cgaggatgtcatcaaggag	tcagtgaggctgtgttaacg
<i>rCOX2</i>	tcaagacagatcagaagcga	tacctgagtgtcttggattg
<i>rTNF<math>\alpha</math></i>	tgatccgagatgtggaactg	cgagcaggaatgagaagagg
<i>rIL-1<math>\beta</math></i>	tgacctatgtgagctgaaag	gggattttgtcttctgt
<i>rcNOS</i>	aacagtggcaacatcaggtcgg	ggtcgatgcacaactgggtgaa
<i>rCGRP</i>	ttggctattgtgcatctgt	gtgtcccagaagccaaga
<i>rTAC1</i>	agcctcagcagttcttggga	agtcttcattgcgcttctt
<i>rTAC2</i>	ggaaggattgctgaaagtgc	gccataagtcccacaaga
<i>rGAPDH</i>	atgactctaccacggcaag	atgactctaccacggcaag

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

specific primers (30  $\mu$ M). Primer concentrations in the final volume of 25  $\mu$ l 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  $\pm$  S.E.M., with sample sizes of at least five rats per group. In the RT-PCR experiments, the ratio between each gene product and glyceraldehyde-3-phosphate dehydrogenase in control animals was considered as 1. Statistical comparisons of unpaired data were performed by the Mann-Whitney *U*-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

**ATB-429 Pharmacokinetics.** Figure 1B demonstrates that, in contrast to mesalamine, 10 mM ATB-429 functions as an H<sub>2</sub>S donor. Although spontaneous release of H<sub>2</sub>S occurred in a phosphate-buffered solution, H<sub>2</sub>S generation was significantly enhanced by incubating ATB-429 with liver homogenates, suggesting that this compound effectively generates H<sub>2</sub>S both by nonenzymatic and enzymatic activities, although the nature of the enzymes involved in this process was not addressed. The amount of H<sub>2</sub>S released in vitro by ATB-429 was significantly higher than that released by ADT-OH alone.

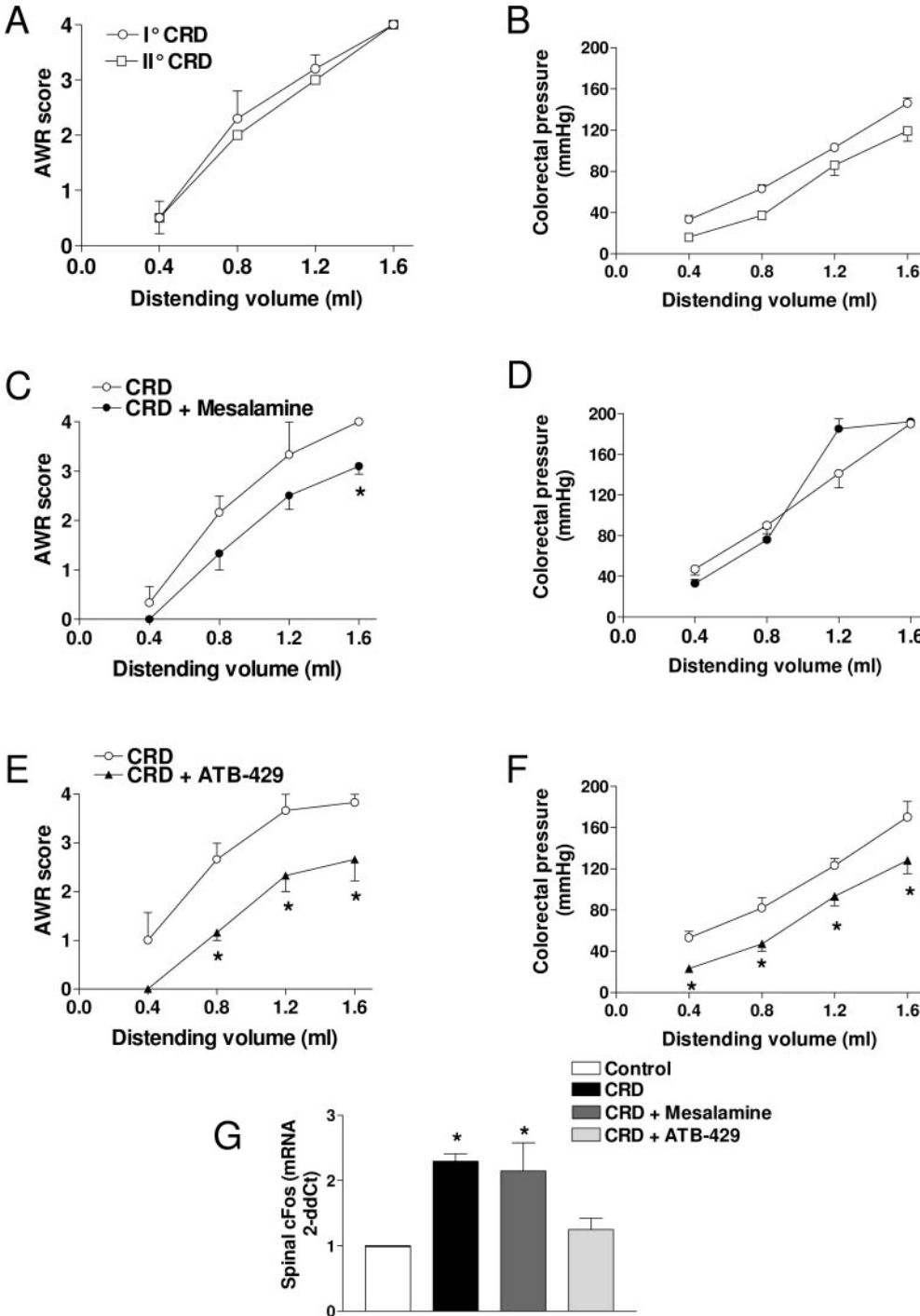
To investigate whether ATB-429 releases H<sub>2</sub>S in vivo, plasma H<sub>2</sub>S concentrations were measured in rats administered 100 mg/kg ATB-429 i.p. (Fig. 1C). ATB-429 increased plasma H<sub>2</sub>S concentrations in a time-dependent manner with a peak occurring 10 min after drug injection ( $n = 4-5$ ; \*,  $P < 0.05$  versus basal), and concentrations returned to basal values 60 min later. Due to this kinetics, all of the distension experiments described hereafter were carried out 10 to 30 min after ATB-429 administration.

**ATB-429 Does Not Induce Colonic Damage.** Macroscopic examination of the colon revealed that the inflammation scores after CRD alone or CRD plus drug 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 mesalamine, ATB-429, or glibenclamide did not significantly affect colonic MPO activity (data not shown).

**ATB-429 Inhibits CRD-Induced Hypersensitivity in Healthy Rats.** In all experiments, two sequential distension-effect curves were constructed. The first distension-effect curve acted as basal, and the second curve was constructed following vehicle or drug administration. In all experiments, rats were conscious, and none of the treatments induced changes in the state of consciousness. CRD 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 increase of the AWR score (less than 1) that was

associated with a small rise of colorectal pressure ( $\approx 20$  mm Hg), indicating that this CRD represents a nonpainful stimulus. Distensions with 1.2 and 1.6 ml of water were associated with greater AWR scores (3 and 4, respectively) and with a high colorectal pressures (up to 80 mm Hg), indicating that these volumes induced noxious sensations (Ji and Traub, 2001). Mesalamine (100 mg/kg i.p.) caused only a slight reduction of the AWR score, reaching statistical significance only at the greatest volume (1.6 ml) (Fig. 2C). It did not affect the colorectal compliance (Fig. 2D).

In contrast, ATB-429 (100 mg/kg i.p.) caused a significant decrease of the AWR response to CRD (Fig. 2E) with a con-

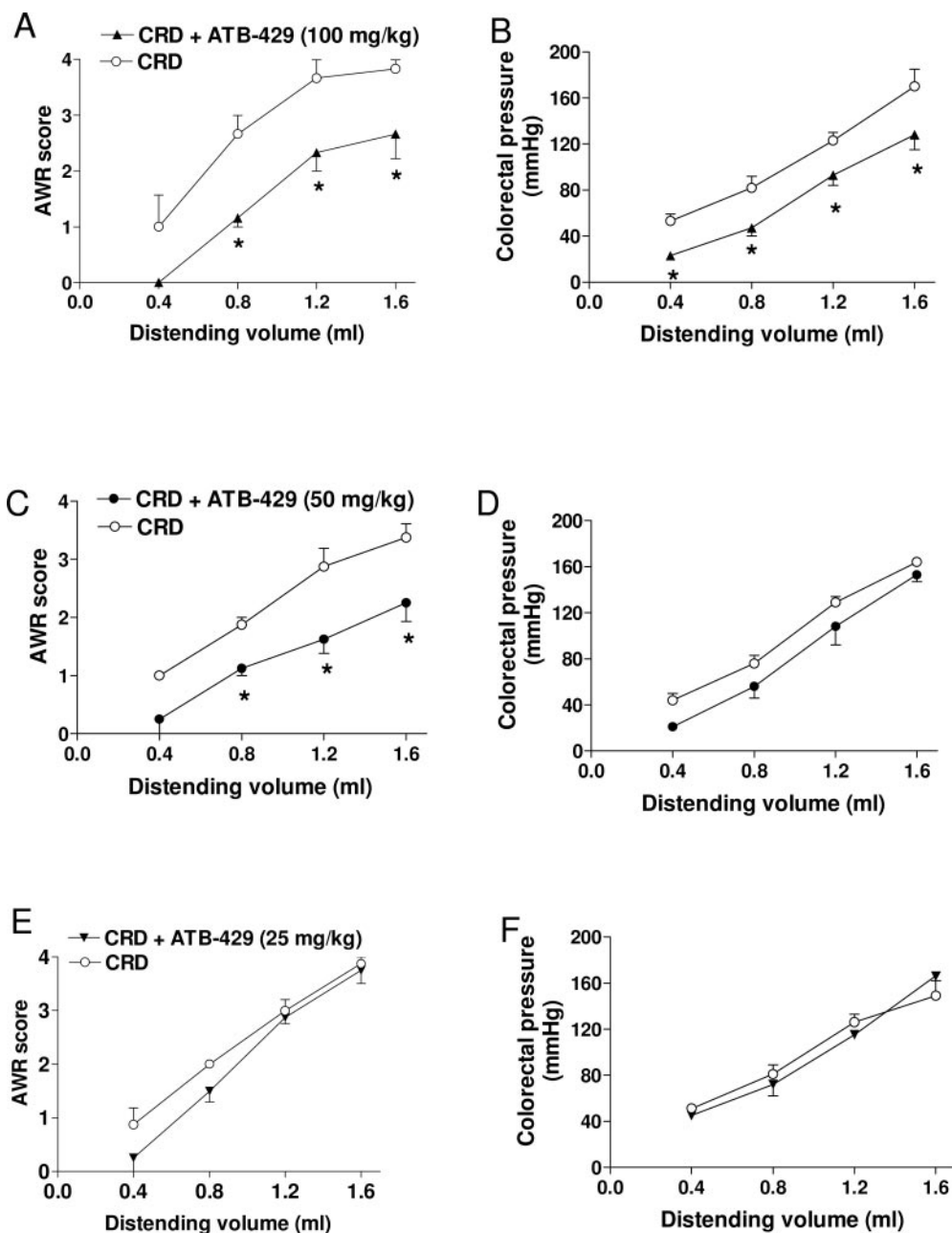


**Fig. 2.** ATB-429 reverses CRD-induced nociception in conscious, healthy rats. CRD induces a volume-dependent, reproducible increase in AWR score (A) without a significant change in colorectal compliance (B). Mesalamine (100 mg/kg i.p.) induces a slight reduction of visceral perception and pain (C) and does not alter the colorectal compliance (D), whereas ATB-429 (100 mg/kg i.p.) significantly reduces visceral perception and pain (E) and causes a relaxation of colonic smooth muscle (F). Data are mean  $\pm$  S.E. of five rats. \*,  $P < 0.05$  versus CRD. The analysis of spinal c-Fos expression confirms the antinociceptive effect of ATB-429 (G). Data are mean  $\pm$  S.E. of five rats. \*,  $P < 0.05$  versus control.

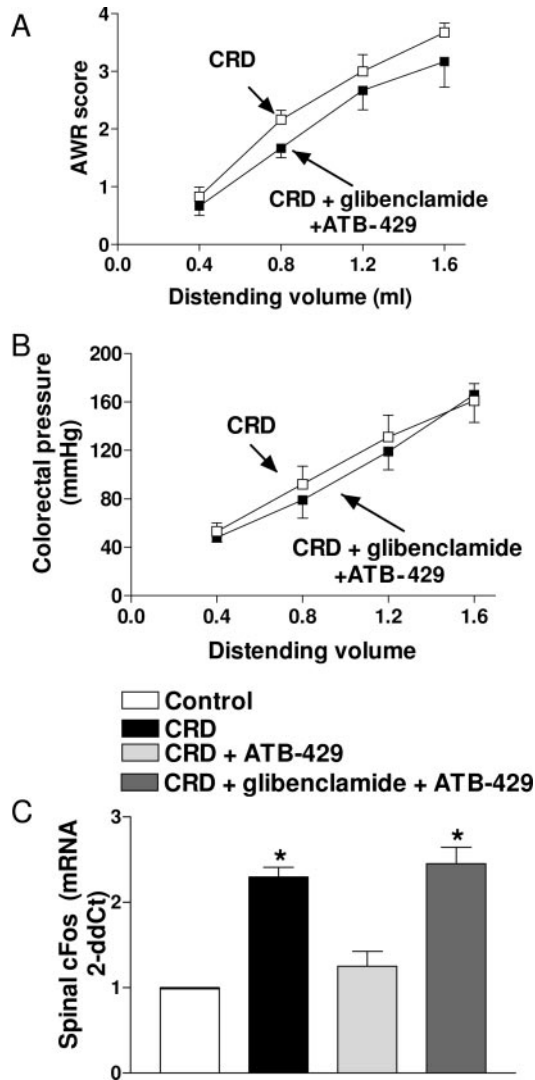
comitant increase in rectal compliance (Fig. 2F). The antinociceptive effects of ATB-429 during CRD were confirmed by analysis of c-Fos expression in the spinal cord. Quantitative RT-PCR of c-Fos mRNA expression demonstrates that CRD induced a 2-fold increase in spinal c-Fos expression that was not modified by mesalamine. Administration with ATB-429 abrogated c-Fos mRNA induction caused by CRD, suggesting that the reduced AWR score was due to the antinociceptive effect of the H<sub>2</sub>S moiety of ATB-429, rather than the mesalamine component of the new molecule (Fig. 2G).

The antinociceptive effect of ATB-429 was dose-dependent, because it was maximal at the dose of 100 mg/kg (Fig. 3A), it was maintained at the dose of 50 mg/kg (Fig. 3C), but it was not apparent at the dose of 25 mg/kg (Fig. 3E). The relaxant effect was demonstrated only at the highest dose (Fig. 3, B, D, and F).

**K<sub>ATP</sub> Channel Blockade Reverses the Antinociceptive Effect of ATB-429.** To determine whether ATP-sensitive K<sup>+</sup> channels were involved in the antinociceptive effects of ATB-429, the interaction of ATB-429 with a known K<sub>ATP</sub> channel modulator was examined. The inhibitory effect of ATB-429 on CRD-induced pain was reversed by pretreating rats with glibenclamide, a K<sub>ATP</sub> channel antagonist (Fig. 4A), and this effect was accompanied by inhibition of colonic smooth muscle relaxation (Fig. 4B). In contrast, treating rats with glibenclamide alone had no effect on CRD-induced nociception or colonic compliance (data not shown). Analysis of c-Fos expression demonstrated that pretreatment with glibenclamide reversed the antinociceptive effect of ATB-429 (Fig. 4C). Plasma H<sub>2</sub>S concentrations did not significantly change during K<sub>ATP</sub> channel modulator pretreatment (data not shown).



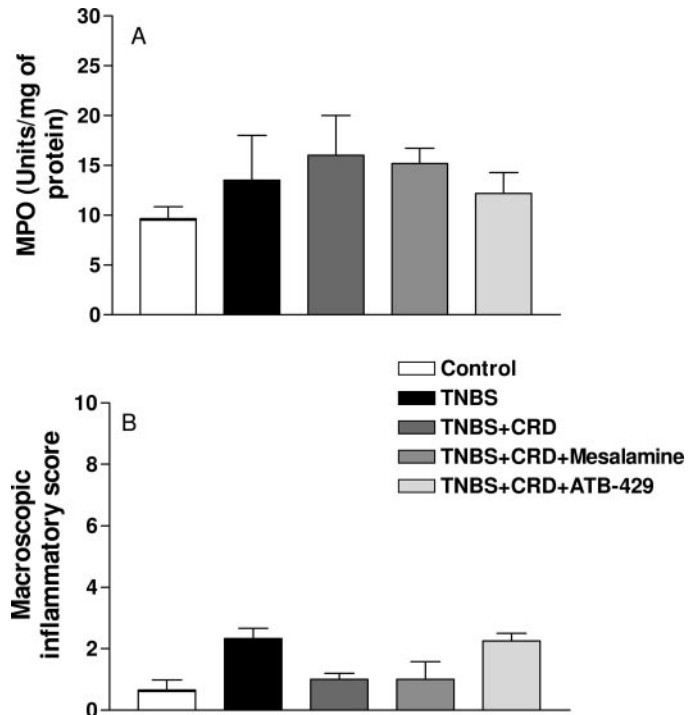
**Fig. 3.** Antinociceptive effect of ATB-429 is dose-dependent. ATB-429 induces a dose-dependent inhibition of the nociceptive action of the CRD that is significant at doses of 100 mg/kg (A) and 50 mg/kg (C), whereas the dose of 25 mg/kg is ineffective (E). At the higher dose, ATB-429 induces significant colorectal relaxation (B), whereas at the doses of 50 mg/kg (D) and 25 mg/kg (F), no change of the colorectal compliance is observed. Data are mean ± S.E. of five rats. \*, *P* < 0.05 versus CRD.



**Fig. 4.** Antinociceptive effect of ATB-429 is modulated by  $K_{ATP}$  channels. Pretreating healthy rats with the  $K_{ATP}$  channel blocker glibenclamide ( $2.8 \mu\text{mol/kg}$  i.v.) abrogates the antinociceptive (A) and myorelaxant (B) effects of ATB-429 ( $100 \text{ mg/kg}$  i.p.). These results are confirmed by quantitative RT-PCR data on spinal c-Fos expression (C). Data are mean  $\pm$  S.E. of five rats. \*,  $P < 0.05$  versus control.

**ATB-429 Inhibits Pain in Allodynic Rats.** Colitic rats exhibited a 20% reduction of body weight compared with healthy rats, and diarrhea was observed during the first week after induction of colitis. Four weeks after induction of colitis, colonic MPO activity was not significantly increased in TNBS-treated rats in comparison with controls (Fig. 5A), indicating that inflammation was almost resolved. These data were confirmed by the analysis of the macroscopic inflammatory score that demonstrated that only thickening of bowel wall was observed in TNBS-treated rats compared with controls, whereas hyperemia and ulceration had disappeared (Fig. 5B).

When CRD was performed 4 weeks after induction of colitis, a significant increase in the AWR score was observed in comparison with healthy rats. As shown in Fig. 6A, an increased nociception was observed during the low-volume (0.4 and 0.8 ml of water) and high-volume (1.2 ml of water) distensions, indicating that colonic inflammation induces allodynia (perception of nonpainful stimulus as painful) and hyperalgesia (perception of painful stimulus as more painful)

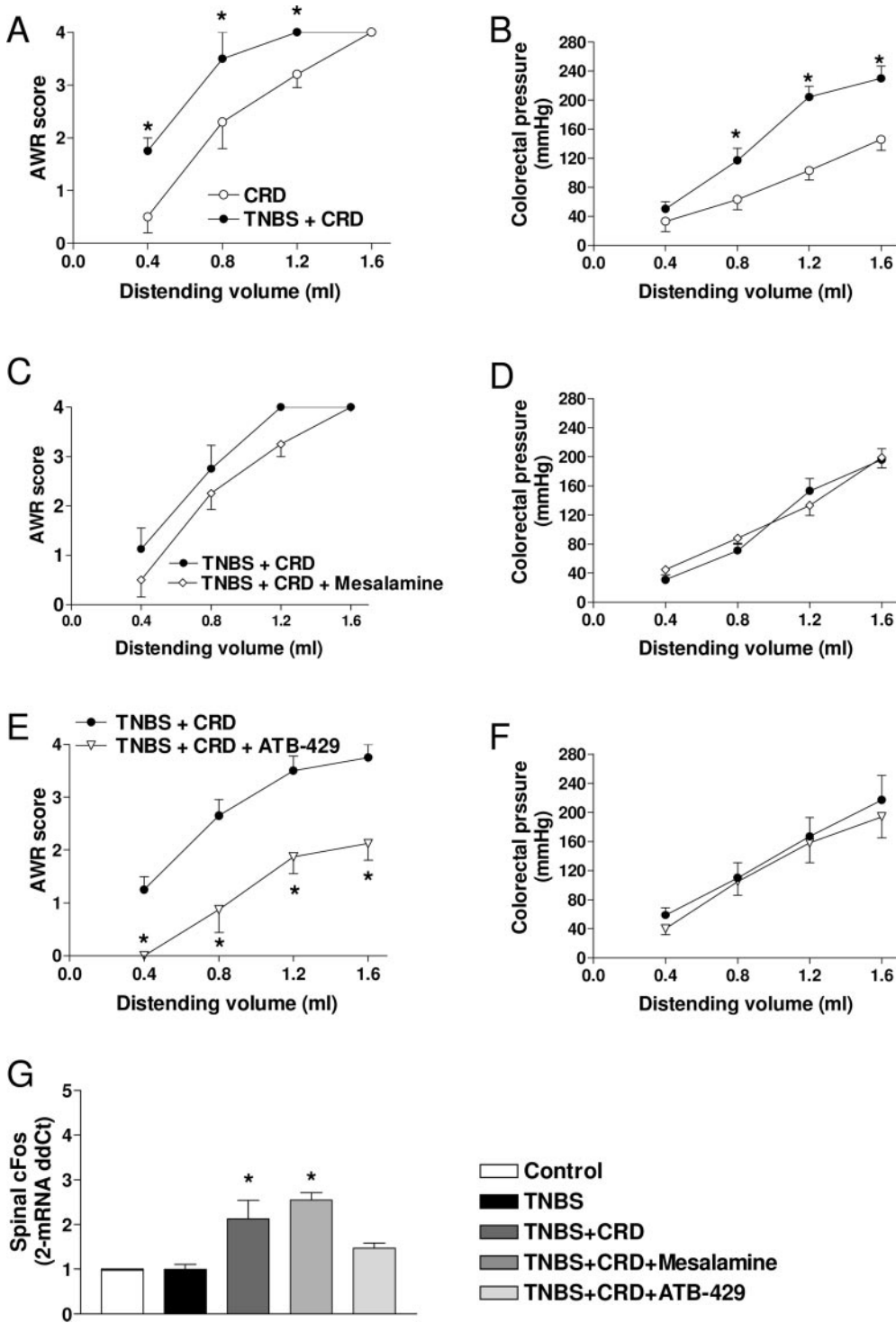


**Fig. 5.** Lack of inflammation after TNBS-induced colitis. Colitis is almost resolved 4 weeks after TNBS administration, as demonstrated by the colonic MPO (A) and macroscopic inflammatory score (B). CRD alone, mesalamine ( $100 \text{ mg/kg}$  i.p.), and ATB-429 ( $100 \text{ mg/kg}$  i.p.) do not modify these two parameters. Data are mean  $\pm$  S.E. of five rats.

to CRD. Interestingly, colonic compliance of postcolitic rats was also significantly lower than that of control animals (Fig. 6B), probably due to the fibrotic evolution of the colitis. The AWR score and colonic compliance in response to repeated CRDs and CRD + mesalamine ( $100 \text{ mg/kg}$  i.p.) did not change (Fig. 6, C and D, respectively), whereas pretreating colitic rats with  $100 \text{ mg/kg}$  i.p. ATB-429 almost completely inhibited the allodynic response to CRD (Fig. 6E) without modifying the colonic compliance (Fig. 6F). The expression of c-Fos mRNA in the spinal cord was greatly increased in the colitic rats after CRD, indicating the presence of a painful condition after induction of colitis. The administration of ATB-429, but not of mesalamine, reduced c-Fos mRNA expression to values similar to that of controls (Fig. 6G).

**Plasma Concentration of  $\text{H}_2\text{S}$ .** In experiments with both healthy (Fig. 7A) and postcolitic (Fig. 7B) rats, plasma concentrations of  $\text{H}_2\text{S}$  significantly increased after ATB-429 administration. In contrast, repeated CRD or mesalamine administration had no effect.

**Colonic and Spinal Cord Gene Expression.** TNBS-induced colonic inflammation resulted in up-regulation of CBS and CSE mRNA, which was not modified by ATB-429 administration, whereas mesalamine down-regulated CBS expression (Fig. 8). Moreover, in postcolitic rats, we observed an overexpression of COX-1, COX-2, TNF $\alpha$ , IL-1 $\beta$ , and cNOS mRNAs that was not modified by CRD or mesalamine administration (Fig. 9), whereas ATB-429 significantly inhibited the colonic expression of COX-2 and IL-1 $\beta$  (Fig. 9, B and D, respectively). TNBS-induced colitis was associated with increased CGRP, TAC-1, and TAC-2 mRNA expression in the colon that was not modified by mesalamine or ATB-429 administration (data not shown).



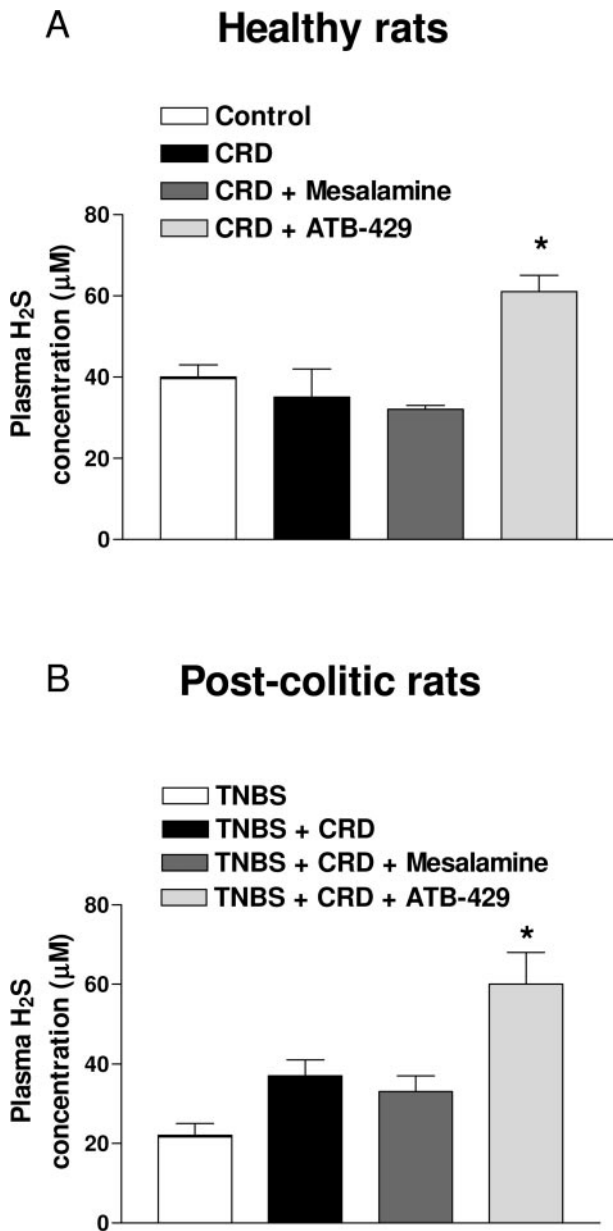
**Fig. 6.** ATB-429 exerts antinociceptive effects in allodynic rats. Colonic inflammation induced by TNBS causes allodynia and hyperalgesia (A) that are completely reversed by ATB-429 at the dose of 100 mg/kg (E), but not by mesalamine administered at the same dose (C). However, in contrast to data obtained in healthy rats, TNBS-induced colitis elicits a significant decrease in colorectal compliance (B) that is not modified by mesalamine (D) or ATB-429 (F). Data are mean ± S.E. of five rats. \*, *P* < 0.05. The antinociceptive effects of ATB-429 are confirmed by RT-PCR analysis of spinal c-Fos mRNA expression (G). Data are mean ± S.E. of five rats. \*, *P* < 0.05 versus control.

TNBS-induced colonic inflammation did not modify spinal cord expression of CBS and elicited only a slight increase in CSE expression (data not shown); CRD and administration of either mesalamine or ATB-429 had no effect on expression of these genes (data not shown). Likewise, in TNBS groups, we did not observe any significant modification of inflammatory gene expression except IL-1β that was reduced by ATB-429, but not mesalamine (data not shown). CRD induced a significant over-expression of spinal COX-2 and TNF-α that was only partially reversed by mesalamine and ATB-429 (data not shown). Fi-

nally, we did not observe any significant modification of spinal CGRP, TAC-1, or TAC-2 mRNA expression after induction of colitis, and CRD, mesalamine, and ATB-429 did not modify expression of these genes (data not shown).

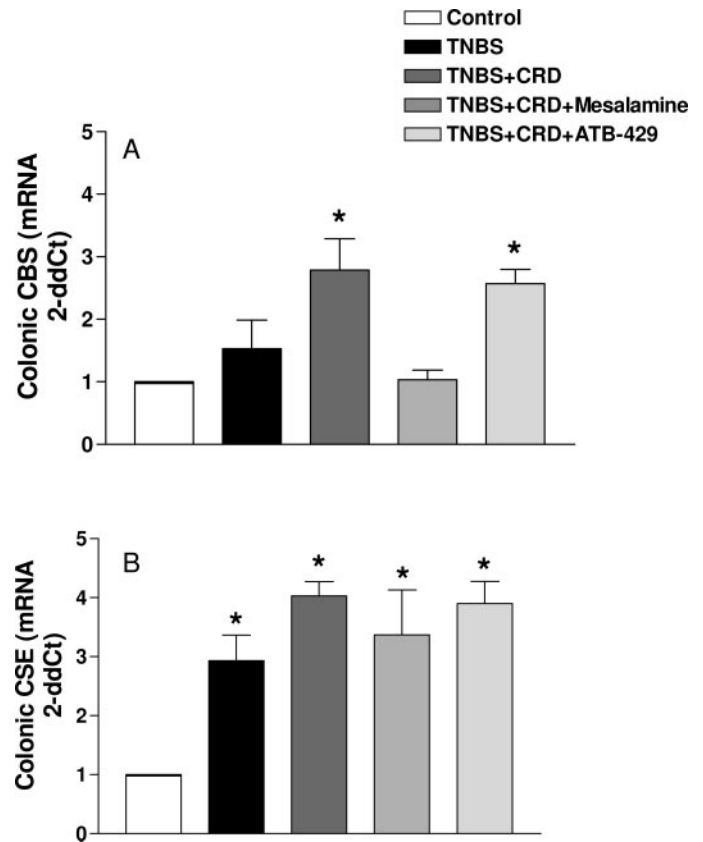
### Discussion

The studies described herein demonstrate that ATB-429, an H<sub>2</sub>S-releasing derivative of mesalamine, functions as an antinociceptor in healthy rats and in a postcolitic model of



**Fig. 7.** ATB-429 increases the plasma concentration of H<sub>2</sub>S. In both healthy (A; data are mean  $\pm$  S.E. of five rats; \*,  $P < 0.05$  versus control) and postcolitic (B; data are mean  $\pm$  S.E. of five rats; \*,  $P < 0.05$  versus TNBS) rats, ATB-429 administration induces a significant increase in the plasma concentrations of H<sub>2</sub>S.

rectal hypersensitivity. These effects are produced, at least in part, through K<sub>ATP</sub> channels. ATB-429 is a new chemical entity consisting of an H<sub>2</sub>S-releasing moiety (a thione group) linked to mesalamine, a known anti-inflammatory agent, and, it is noteworthy that a 100-mg/kg dose of ATB-429 delivers only ~38 mg/kg mesalamine. Here, we provide evidence that ATB-429 is significantly more effective than mesalamine in reducing nociception caused by colonic distension in intact and allodynic rats. The antinociceptive action of the ATB-429 was dose-dependent, and at the higher doses tested (50 and 100 mg/kg), it significantly decreased the AWR score following repetitive noxious and non-noxious CRD. Antinociceptive activities of ATB-429 did not correlate with changes in colorectal compliance, because a significant decrease of colorectal compliance was observed only with the

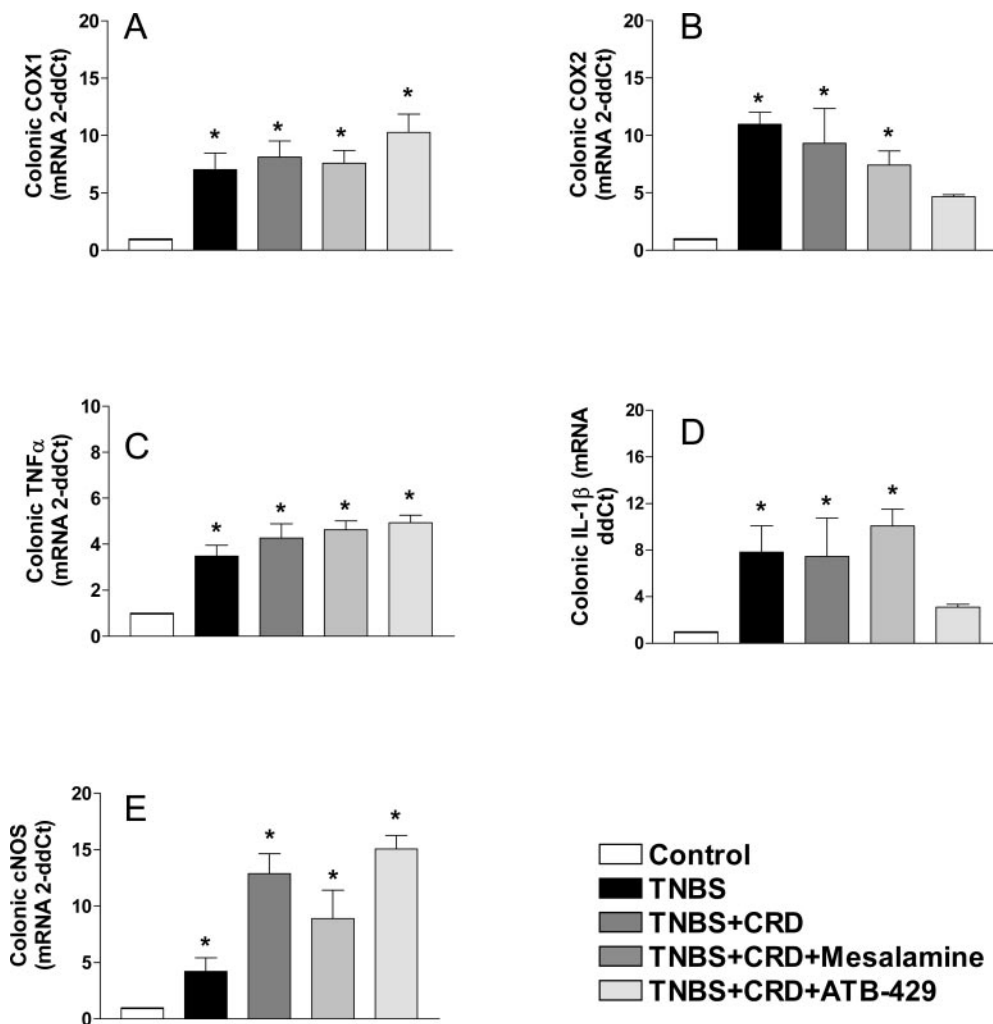


**Fig. 8.** Colonic CBS and CSE are modulated by inflammation. Colitis induces an overexpression of both CSE (A) and CBS (B). Although mesalamine (100 mg/kg i.p.), but not ATB-429 (100 mg/kg i.p.), reverses the increase in CBS expression, neither mesalamine nor ATB-429 inhibited the overexpression of CSE. Data are mean  $\pm$  S.E. of five rats. \*,  $P < 0.05$  versus control.

highest dose (100 mg/kg), whereas lower doses had no relaxant effect on colonic smooth muscle cells.

Several of our data support the hypothesis that the antinociceptive effects of ATB-429 are due to its H<sub>2</sub>S moiety. First, mesalamine itself was only marginally effective in controlling pain in the model. Second, ATB-429, but not mesalamine, releases H<sub>2</sub>S in vitro, and it does the same in vivo, with a peak of plasma H<sub>2</sub>S concentration occurring rapidly (10–30 min) after i.p. administration (100 mg/kg). Third, two pharmacological actions of ATB-429, antinociception and reduction of c-Fos expression in the spinal cord, were reversed by glibenclamide, a K<sub>ATP</sub> channel inhibitor (Edwards and Weston, 1993; Distrutti et al., 2006). Several H<sub>2</sub>S activities have been shown to be mediated via K<sub>ATP</sub> channels (Wang, 2002), and this finding is consistent with our previous observation demonstrating that antinociception exerted by H<sub>2</sub>S is glibenclamide-sensitive (Distrutti et al., 2006). K<sub>ATP</sub> channels are expressed in many excitable cells, including skeletal and smooth muscle cells as well as neurons from both central and peripheral nervous system. Because these receptors are not discriminated by glibenclamide, the use of this agent does not allow for identification of the site of action of ATB-429 (central versus peripheral) and deserves further investigation.

The antinociceptive action of ATB-429 is maintained in a rodent model of postinflammatory pain. In animal models of acute (Bonaz et al., 2000) and chronic (Julia et al., 1995)



**Fig. 9.** Induction of colonic proinflammatory genes by inflammation is down-regulated by ATB-429. Colitis is associated with an overexpression of several genes involved in the inflammatory response. ATB-429 (100 mg/kg i.p.) down-regulated the colonic expression of COX-2 (B) and IL-1 $\beta$  (D), whereas mesalamine (100 mg/kg i.p.) had no effect. Data are mean  $\pm$  S.E. of five rats. \*,  $P < 0.05$  versus control.

inflammation, abnormal pain responses to CRD have been observed, demonstrating that inflammation induces both hyperalgesia and allodynia that persist when local inflammation is partially or totally resolved. Human studies in patients with ulcerative colitis and Crohn's disease (Bernstein et al., 1996; Chang et al., 2000) and IBS (Collins et al., 2001) have confirmed that colonic inflammation modulates colonic neural afferents. In the present study, an elevated AWR score was observed in postcolitic rats in response to low-volume distension (0.4 and 0.8 ml of water), confirming that TNBS-induced inflammation causes allodynia. Moreover, c-Fos mRNA expression was increased in postcolitic rats in comparison with healthy controls, suggesting that colonic inflammation activates a population of second order spinal cord neurons (Traub et al., 1992). Of interest, ATB-429 completely reversed the allodynic effect of colonic inflammation and down-regulated c-Fos mRNA expression in the spinal cord.

Several inflammatory and noninflammatory mediators are thought to be involved in the hyperalgesia and/or allodynia observed in postinflammatory states. Here, we found that colonic expression of COX-1, COX-2, TNF- $\alpha$ , IL-1 $\beta$ , cNOS, CGRP, TAC-1, and TAC-2 increased in postcolitic rats in comparison with healthy rats. Previous studies have associated the increased expression of these mediators with development of hypersensitivity, and their mechanistic role has

been investigated by pharmacological and genetic approaches (Abbadie, 2005). The development of hypersensitivity in postinflammatory states supports the notion that inflammation induces long-lasting changes in the mechanisms underlying visceral pain. Structural and molecular changes take place in the colon of IBS patients and along with the demonstration that IBS-like symptoms develop in postinfectious colitis, these data support the notion that intestinal inflammation plays a crucial role in precipitating IBS symptoms in susceptible individuals (Sartor, 1994). Further supporting the link between inflammation and IBS, IL-1 $\beta$  has been found to be elevated in the colonic mucosa of a subset of diarrhea-predominant IBS patients (Sartor, 1994), and COX-2 is up-regulated in animal model in which transient acute infection leads to persistent muscle hypercontractility (Akiho et al., 2005). Moreover, it has been demonstrated that IL-1 $\beta$  induces spinal COX-2 up-regulation and pain hypersensitivity following peripheral inflammation (Lee et al., 2004). In the present study, we have shown that colon expression of IL-1 $\beta$  and COX-2 mRNA was persistently increased after TNBS-induced inflammation and that ATB-429, but not mesalamine, reduced the colonic expression of these mediators. Previous studies have shown that H<sub>2</sub>S exerts anti-inflammatory activities and, similar to NO, reduces neutrophil adherence to endothelial cells in the mesenteric circulation (Fiorucci et al., 2005), suggesting that the H<sub>2</sub>S-

releasing moiety contribute to the anti-inflammatory effects of this compound (Fiorucci et al., 2006).

The mechanism(s) through which ATB-429 exerts its antinociceptive activities remains to be identified, although several explanations could be taken into consideration. First, because high concentrations of H<sub>2</sub>S are neurotoxic, one might speculate that H<sub>2</sub>S released by ATB-429 alters rat consciousness, a situation that mimics a pain-free condition during CRD (Distrutti et al., 2006). This explanation, however, is unlikely, because concentrations required for neurotoxic effects by H<sub>2</sub>S are significantly higher than that measured in our experimental setting. Plasma and brain levels of H<sub>2</sub>S in healthy rats range from 10 to 160 μM (Wang, 2002), whereas neurotoxic effects (inhibition of synaptic transmission in the hippocampus) occur at concentrations >320 μM (Abe and Kimura, 1996). Not only did ATB-429 have no effect on the rat consciousness but also plasma H<sub>2</sub>S concentrations measured in rats administered 100 mg/kg ATB-429 (the higher dose used in this study) never exceed 60 to 70 μM. Second, because H<sub>2</sub>S causes smooth muscle relaxation (Zhao et al., 2001), the antinociceptive activities of ATB-429 might be due to an increase in colorectal compliance. However, this is also an unlikely explanation, because ATB-429 maintains its analgesic action also at a dose (50 mg/kg) that fails to decrease colorectal tone. Moreover, in the postcolitic model in which ATB-429 is powerfully analgesic, the colorectal compliance that is persistently reduced in comparison with healthy rats as a consequence of colonic fibrosis induced by TNBS is not affected by this agent. Third, ATB-429 might modulate inflammation in the colon. Although inflammation results in enhanced nociception and its reversal attenuates perception of painful stimuli, and ATB-429 exerts anti-inflammatory activities in rodent models of colitis, it is unlikely that anti-inflammation by itself explains all its antinociceptive activities. In support to this concept, we have shown that 1) ATB-429 is antinociceptive also in healthy rats, where no inflammation is detectable; 2) in both healthy and postcolitic rats, ATB-429 is antinociceptive after a single dose; and 3) in the postcolitic model, ATB-429 was administered 4 weeks after the induction of colitis, when the inflammatory process was largely resolved. Fourth, a likely explanation of the antinociceptive activity of ATB-429 might deal, therefore, with the ability of ATB-429 to modulate neurotransmission of painful stimuli. Support to this concept comes from the observation that antinociceptive activity of ATB-429 associates with significantly inhibition of CRD-induced spinal c-Fos expression. Because induction of this gene by CRD is a marker of activation of second order spinal cord neurons (Traub et al., 1992), its reversal by ATB-429 supports a direct modulatory function on these neurons. The findings that mesalamine fails to modulate c-Fos expression in this experimental setting and that effects of ATB-429 on c-Fos are reversed by glibenclamide support a role for K<sub>ATP</sub> channels on the afferent, sensitive spinal fibers.

In summary, we have shown that systemic administration of ATB-429 reduces visceral sensitivity and pain perception in conscious healthy and postcolitic, hypersensitive, rats. The neurophysiological basis for these actions seems to be dependent on the H<sub>2</sub>S-releasing moiety of ATB-429 and might involve K<sub>ATP</sub> channels on afferent, sensitive spinal fibers. In addition, ATB-429 modulates expression of colonic proinflammatory mediators such as COX-2 and IL-1β. Whether

H<sub>2</sub>S-releasing drugs will have utility in the treatment of painful functional and organic intestinal diseases in humans remains to be investigated.

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**Address correspondence to:** Dr. Eleonora Distrutti, Clinica di Gastroenterologia, Policlinico Monteluce, University of Perugia, Via Enrico Dal Pozzo, 06122 Perugia, Italy. E-mail: eleonoradistrutti@katamail.com

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