JPET Fast Forward. Published on July 19, 2006 as DOI: 10.1124/jpet.106.106435
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Title Page

ATB-429, a hydrogen sulfide-releasing derivative of mesalamine, exerts anti-nociceptive effects in a model of post-inflammatory hypersensitivity

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Running title page

Running title: ATB-429 inhibits visceral hypersensitivity

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Document statistics

Text pages 35

Figures 9

Table 1

References 40

Words in the Abstract 241

Words in the Introduction 718

Words in the Discussion 1353

Non-standard abbreviation: AWR, abdominal withdrawal reflex; CRD, colorectal distension; IBS, irritable bowel syndrome.

Recommended section: Gastrointestinal, hepatic, pulmonary and renal.

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Abstract

Hydrogen sulfide (H_2S) 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_2S-releasing derivative of mesalamine (ATB-429) in modulating nociception to colorectal distension (CRD), a model that mimics some features of IBS, in healthy and post-colitic rats. Four, graded (0.4-1.6 ml water) CRD were produced in conscious rats and colorectal sensitivity and pain were assessed by measuring the abdominal withdrawal response (AWR) 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 cFOS mRNA, while mesalamine had no effect. ATB-429-induced anti-nociception was reversed by glibenclamide, a K_{ATP} channel inhibitor. The antinociceptive effect of ATB-429 was maintained in a rodent model of post-inflammatory hypersensitivity (4 weeks after colitis induction). At a dose of 100 mg/kg, ATB-429 reversed the allodynic response caused by CRD in post-colitic rats. Colonic COX-2 and IL-1β mRNA and spinal cFOS mRNA expression were significantly down-regulated by ATB-429, but not by mesalamine. ATB-429, but not mesalamine, increased blood concentrations of H_2S in both healthy and post-colitic rats. Taken together these data suggest that ATB-429 inhibits hypersensitivity induced by CRD in both healthy and post-colitic, allodynic rats by a K_{ATP} channel-mediated mechanism. This study provides evidence that H_2S-releasing drugs might have beneficial effects in the treatment of painful intestinal disorders.

Introduction

Irritable bowel syndrome (IBS) is a common disorder involving the gastrointestinal tract usually defined by the coexistence 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 allodynia 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, 2002). Similarly, low grade inflammation is found in the colon of IBS patients and 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 post-infective IBS (Garcia-Rodriguez and Ruigomez, 1999) and, *second,* histology 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 post-inflammatory states, immune cells localize in the close 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

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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 (IBD) develop IBS-like symptoms and visceral hyperalgesia during the quiescent phases of their diseases (Rao et al., 1987; Isgar et al., 1983). 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). 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 (Wang, 2002; Moore et al., 2003). H_2S 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_2S may act as a pro-inflammatory mediator (Li et al., 2005; Bathia et al., 2005). 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 (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^+ (K_{ATP}) channels.

We have recently shown that cystathionine-β synthase (CBS) and cystathionine-γ lyase (CSE), the two major enzymes involved in H_2S generation, are constitutively expressed in colon and spinal cord and that measurable quantities of H_2S are produced by these tissues. Moreover, H_2S administration inhibits colorectal distension (CRD)-induced pain in healthy and allodynic rats by acting on the K_{ATP} 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_2S-releasing moiety with mesalamine (5-amino salicylic acid). Here we have compared the effects of ATB-429 to those of mesalamine in terms of alleviating CRD-induced hypersensitivity in healthy and post-colitic rats.

Methods

Structure and purity of ATB-429.

The chemical structure of 5-amino-2-hydroxy-benzoic acid 4-(5-thioxo-5H-[1,2] dithiol-3-yl)-phenyl ester (ATB-429) is illustrated in Figure 1 while key synthetic steps are shown in Supplemental Figure 1. The structure of the ATB-429 was verified spectroscopically by proton ^{1}H-NMR and ^{14}C-NMR. Spectra were recorded on Varian Mercury Plus 400 instrument (Varian, Torino, Italy). Chemical shifts are referred to Me_4Si as internal standard. Mass spectra of the synthesized products were performed on API 2000 mass spectrometry (Applayed Biosystem International, Monza, Italy). Melting point was performed on Buchi B-540 instrument (BÜCHI Labortechnik AG, Switzerland). Purity of the compound was 98%, as assessed by HPLC (Varian, Torino, Italy). The 5-p-hydroxyphenyl-1,2-dithione-3-thione (ADT-OH) (Christen MO, 1995) was used as H_2S-releasing moiety (Figure 1A).

Materials

Ascorbic acid, salicylic acid, potassium hydroxide, L-cysteine, N-acetyl-L-cysteine, DL-propargylglycine, trichloroacetic acid, pyridoxal-5'-phosphate, poly ethylene glycol (PEG), glibenclamide, mesalamine, phosphate buffered saline were purchased from Sigma-Aldrich (S. Louis, MO, USA). ADT-OH and ATB-429 were provided by Antibe Therapeutics Inc. (Calgary, Canada). 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 (DI) 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 07:00. 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 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 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 increments of 0.4 ml starting from 0.4 ml and increasing to 1.6 ml water. Animals underwent a double set of CRD. Ten minutes after the first CRD (0.4-1.6 ml 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 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 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 (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 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, while the CRD group consisted of fasting, healthy animals that underwent to 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_{ATP} channels in the modulation of visceral perception by H_2S was assessed by pre-treating rats with glibenclamide (K_{ATP} channel blocker) at a dose of 2.8 µmol/kg i.v. for 20 minutes 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 (L1-L5) were collected for further analysis.

Induction of colitis

Colitis was induced as previously described (Wallace et al., 1989). Briefly, rats were anesthetized with pentobarbital (60 mg/kg i.p.). 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 myeloperoxidase (MPO) activity, an index of granulocyte infiltration, as previously described (Santucci et al., 1995).

***In vitro* H_2S release.**

To compare the *in vitro* H_2S release induced by mesalamine, ATB-429 and ADT-OH, the H_2S releasing moiety of ATB-429, 100-150 mg of isolated livers were homogenized in 1 ml of ice- cold T-PER protein extractor. The H_2S release was lead on the same reactor of plasma analysis. Two ml of an assay reaction mixture was introduced in the reactor.

The mixture contained 10 mM ATB-429 or 10 mM ADT-OH dissolved in PEG 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 in the second reactor containing 2 ml of SAOB as described previously. 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 previously described (Ubuka, 2002; Khan et al., 1980).

Measurement of plasma H_2S concentrations

To determine the kinetics of H_2S released from ATB-429, groups of 4-5 rats were treated with ATB-429 at the dose of 100 mg/kg i.p. and sacrificed after 10, 30, 60 and 180 minutes. A time-course curve of plasma H_2S concentrations was then constructed. Plasma H_2S concentrations were measured as described previously (Ubuka, 2002; Zhao et al., 2001) with modifications. Briefly, 250 µl of plasma were added to ice-cold 250 µl of NaOH 0.1 N in a sealed 3-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_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

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(Model 9616 S^{2-}/Ag^{+} electrode, Orion Research, Beverly, MA, USA) and expressed as H_2S (Ubuka, 2002; Khan et al., 1980).

RT-PCR 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, cyclo-oxygenase (COX)-1 and 2, tumor necrosis factor (TNF)-α, Interleukin (IL)-1β, constitutive NO synthase (cNOS), Calcitonin Gene Related Peptide (CGRP), Takikinin (TAC)-1 and 2 in post-colitic rats. Briefly, 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 µg RNA was added to mixture which contains 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 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 synthesized by MWG BIOTECH and designed using software PRIMER3-NEW using published sequence data from the NCBI database. Table 1 illustrates the rat primers (sense and antisense) used in this study. 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. In the RT-PCR experiments, the ration between each gene product and GAPDH in control animals was considered as 1. Statistical comparisons of unpaired data were performed by the Mann-Whitney test, while statistical comparisons of paired data were performed by the Wilcoxon signed rank test. An associated probability (p value) of less that 5% was considered significant.

Results

ATB-429 pharmacokinetics

Figure 1B demonstrates that, in contrast to mesalamine, ATB-429 (10 mM) functions as an H_2S donor. While spontaneous release of H_2S occurred in a phosphate buffer solution, H_2S generation was significantly enhanced by incubating ATB-429 with liver homogenates, suggesting that this compound effectively generates H_2S both by non-enzymatic and enzymatic activities, though the nature of the enzymes involved in this process was not addressed. The amount of H_2S released in vitro by ATB-429 was significantly higher than that released by ADT-OH alone.

To investigate whether ATB-429 releases H_2S in vivo, plasma H_2S concentrations were measured in rats administered 100 mg/kg ATB-429 i.p. (Figure 1C). ATB-429 increased plasma H_2S concentrations in a time-dependent manner with a peak occurring 10 min after drug injection (n=4-5; $P<0.05$ versus basal), and returned to basal values 60 min later. Due to this kinetic all the distension experiments described thereafter were carried out 10-30 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 drugs 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 (Figure 2A) with no significant reduction in colorectal pressure (Figure 2B). Distensions with 0.4 ml water induced a slight increase 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. Distensions with 1.2 and 1.6 ml water were associated with greater AWR scores (3 and 4 respectively) and with a high colorectal pressures (up to 80 mmHg), 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) (Figure 2C) and did not affect the colorectal compliance (Figure 2D).

In contrast, ATB-429 (100 mg/kg i.p.) caused a significant decrease of the AWR response to CRD (Figure 2E) with a concomitant increase in rectal compliance (Figure 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 cFos mRNA expression demonstrates that CRD induced a two-fold increase in spinal c-Fos expression, that was not modified by mesalamine. Administration with ATB-429 abrogated cFOS mRNA induction caused by CRD, suggesting that the reduced AWR score was due to the antinociceptive effect of the H_2S moiety of ATB-429, rather than the mesalamine component of the new molecule (Figure 2G).

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

K_{ATP} channel blockade reverses the antinociceptive effect of ATB-429

To determine if ATP-sensitive K^+ channels were involved in the antinociceptive effects of ATB-429, the interaction of ATB-429 with a known K_{ATP} channel modulator was examined. The inhibitory effect of ATB-429 on CRD-induced pain was reversed by pre-treating rats with glibenclamide, a K_{ATP} channel antagonist (Figure 4A) and this effect was accompanied by inhibition of colonic smooth muscle relaxation (Figure 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 pre-treatment with glibenclamide reversed the antinociceptive effect of ATB-429 (Figure 4C). Plasma H_2S concentrations did not significantly change during K_{ATP} channel modulator pre-treatment (data not shown).

ATB-429 inhibits pain in allodynic rats

Colitic rats exhibited a 20% reduction of body weight when 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 (Figure 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, while hyperemia and ulceration were disappeared (Figure 5B).

When CRD was performed four weeks after induction of colitis, a significant increase in the AWR score was observed in comparison with healthy rats. As shown in Figure 6A, an increased nociception was observed during the low volume (0.4 and 0.8 ml water) and high volume (1.2 ml water) distensions, indicating that colonic inflammation induces allodynia (perception of non painful stimulus as painful) and hyperalgesia (perception of painful stimulus as more painful) to CRD. Interestingly, colonic compliance of post-colitic rats was also significantly lower than that of control animals (Figure 6B), likely due to the fibrotic evolution of the colitis. The AWR score and colonic compliance in response to repeated CRDs and CRD + mesalamine (100 mg/kg i.p.) did not change (Figures 6C and 6D respectively), while pre-treating colitic rats with ATB-429 (100 mg/kg i.p.) almost completely inhibited the allodynic response to CRD (Figure 6E) without modifying the colonic compliance (Figure 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 cFOS mRNA expression to values similar to that of controls (Figure 6G).

Plasma concentration of H_2S

In experiments with both healthy (Figure 7A) and post-colitic (Figure 7B) rats, plasma concentrations of H_2S significantly increased after ATB-429 administration, while repeated CRD or mesalamine administration had no effect.

Colonic and spinal cord gene expression

TNBS-induced colonic inflammation resulted in upregulation of CBS and CSE mRNA, which was not modified by ATB-429 administration, while mesalamine downregulated CBS expression (Figure 8). Moreover, in post-colitic rats we observed an overexpression of COX-1, COX-2, TNFα, IL-1β and cNOS mRNAs that was not modified by CRD or

mesalamine administration (Figure 9), while ATB-429 significantly inhibited the colonic expression of COX-2 and IL-1β (Figures 9B and 9D respectively). TNBS-induced colitis was associated with increased CGRP, TAC1 and TAC2 mRNA expression in the colon that was not modified by mesalamine or ATB-429 administration (data not shown).

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). Similarly, 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 overexpression of spinal COX-2 and TNFα that was only partially reversed by mesalamine and ATB-429 (data not shown). Finally, we did not observe any significant modification of spinal CGRP, TAC1 or TAC2 mRNA expression after induction of colitis, and CRD, mesalamine and ATB-429 did not modify expression of these gene (data not shown).

Discussion

The studies described herein demonstrate that ATB-429, an H_2S-releasing derivative of mesalamine, functions as an antinociceptor in healthy rats and in a post-colitic model of rectal hypersensitivity. These effects are produced, at least in part, through K_{ATP} channels. ATB-429 is a new chemical entity consisting of an H_2S 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 of 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 anti-nociceptive 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. Anti-nociceptive activities of ATB-429 did not correlate with changes in colorectal compliance, since a significant decrease of colorectal compliance was observed only with the highest dose (100 mg/kg), while 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 is due to its H_2S moiety. *First*, mesalamine itself was only marginally effective in controlling pain in the model. *Second*, ATB-429, but not mesalamine, releases H_2S *in vitro*, and it does the same *in vivo*, with a peak of plasma H_2S concentration occurring rapidly (10-30 min) after i.p. administration (100 mg/kg). *Third*, two pharmacological actions of ATB-429, anti-nociception and reduction of c-Fos expression in the spinal cord, were reversed by glibenclamide, a K_{ATP} channel inhibitor (Edwards and Weston, 1993; Distrutti et al., 2006). Several H_2S activities have been shown to be mediated via K_{ATP} channels (Wang, 2002) and this finding is consistent with our previous observation demonstrating that antinociception exerted by H_2S is glibenclamide sensitive (Distrutti et al., 2006). K_{ATP} channels are expressed in many excitable cells including skeletal and smooth muscle cells

as well as neurons from both central and peripheral nervous system. Since these receptors are not discriminated by glibenclamide, the use of this agent does not allow to identify the site of action of ATB-429 (central versus peripheral) and deserves further investigations.

The antinociceptive action of ATB-429 is maintained in a rodent model of post-inflammatory pain. In animal models of acute (Bonaz et al., 2000) and chronic (Julia et al., 1995) 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 post-colitic rats in response to low volume distension (0.4 and 0.8 ml water), confirming that TNBS-induced inflammation causes allodynia. Moreover, c-Fos mRNA expression was increased in post-colitic 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 downregulated cFos mRNA expression in the spinal cord.

Several inflammatory and non-inflammatory mediators are thought to be involved in the hyperalgesia and/or allodynia observed in post-inflammatory states. Here we found that colonic expression of COX-1, COX-2, TNFα, IL-1β, cNOS, CGRP, TAC1 and TAC2 increased in post-colitic 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 post-inflammatory 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 post-infectious 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-1β has been found to be elevated in the colonic mucosa of a subset of diarrhea-predominant IBS patients (Sartor, 1994) and COX-2 is upregulated in animal model in which transient acute infection leads to persistent muscle hypercontractility (Akiho et al., 2005). Moreover, it has been demonstrated that IL-1β induces spinal COX-2 upregulation and pain hypersensitivity following peripheral inflammation (Lee et al., 2004). In the present study we have shown that colon expression of IL-1β 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_2S exerts anti-inflammatory activities and, similarly to NO, reduces neutrophil adherence to endothelial cells in the mesenteric circulation (Fiorucci et al., 2005), suggesting that the H_2S releasing moiety contribute to the anti-inflammatory effects of this compound (Fiorucci et al., 2006).

The mechanism(s) through which ATB-429 exerts its anti-nociceptive activities remains to be identified, although several explanations could be taken into consideration. *First*, because high concentrations of H_2S are neurotoxic, one might speculate that H_2S 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, since concentrations required for neurotoxic effects by H_2S are significantly higher than that measured in our experimental setting. Plasma and brain levels of H_2S in healthy rats range from 10 to 160 μM (Wang, 2002), while neurotoxic effects (inhibition of synaptic transmission in the hippocampus) occurs at concentrations >320 μM (Abe and Kimura, 1996). Not only ATB-429 had no effect on the rats consciousness, but plasma H_2S

concentrations measured in rats administered 100 mg/kg ATB-429 (the higher dose used in this study), never exceed 60-70 μM. *Second,* since H_2S causes smooth muscle relaxation (Zhao et al., 2001), the anti-nociceptive activities of ATB-429 might be due to an increase in colorectal compliance. However, this is also an unlikely explanation since ATB-429 maintains its analgesic action also at a dose (50 mg/kg) that fails to decrease colorecyal tone. Moreover, in the post-colitic 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. While 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 anti-nociceptive activities. In support to this concept we have shown that: a) ATB-429 is anti-nociceptive also in healthy rats, where no inflammation is detectable; b) in both healthy and post-colitic rats ATB-429 is anti-nociceptive after a single dose; c). in the post-colitic model, ATB-429 was administered 4 weeks after the induction of colitis, when the inflammatory process was largely resolved. *Forth*, a likely explanation of the anti-nociceptive 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 anti-nociceptive activity of ATB-429 associates with significantly inhibition of CRD-induced spinal c-Fos expression. Since 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_{ATP} 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 post-colitic, hypersensitive, rats. The neurophysiological basis for these actions appear to be dependent on the H_2S releasing moiety of ATB-429 and might involve K_{ATP} channels on afferent, sensitive, spinal fibers. In addition, ATB-429 modulates expression of colonic pro-inflammatory mediators such as COX-2 and IL-1β. Whether H_2S-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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Footnotes

Dr. Wallace 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.

Drs. Caliendo, Santagada, Cirino, Wallace and Fiorucci hold shares in Antibe Therapeutics Inc., the manufacturer of ATB-429.

Legends for figures

Figure 1

ATB-429 functions as an H_2S donor. Panel A. Chemical structure of ATB-429 showing a mesalamine moiety linked to the H_2S-releasing moiety (ADT-OH). Panel B. ATB-429 releases a H_2S in vitro. Data are ± SE of 5 experiments. *P<0.05. Panel C. Time-course of plasma H_2S concentrations in rats administered ATB-429 (100 mg/kg) i.p. Groups of 4-5 rats were administered ATB-429 (100 mg/kg) at time 0, and sacrificed at indicated time-points. H_2S concentrations in the blood were measured as described in Materials and Methods. Data are mean ± SE. * P<0.05 versus baseline.

Figure 2

ATB-429 reverses CRD-induced nociception in conscious, healthy rats. CRD induces a volume-dependent, reproducible increase in abdominal withdrawal response (AWR; panel A) without a significant change in colorectal compliance (panel B). Mesalamine (100 mg/kg i.p.) induces a slight reduction of visceral perception and pain (panel C) and does not alter the colorectal compliance (panel D), while ATB-429 (100 mg/kg i.p.) significantly reduces visceral perception and pain (panel E) and causes a relaxation of colonic smooth muscle (panel F). Data are mean ± SE of 5 rats. *P<0.05 versus CRD. The analysis of spinal cFos expression confirms the antinociceptive effect of ATB-429 (panel G). Data are mean ± SE of 5 rats. *P<0.05 versus control.

Figure 3

The 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 (panel A) and 50 mg/kg (panel C), while the dose of 25 mg/kg is ineffective (panel E). At the higher dose ATB-429 induces significant colorectal relaxation (panel B), while at the doses of 50 mg/kg (panel D) and 25 mg/kg (panel F) no change of the colorectal compliance is observed. Data are mean ± SE of 5 rats. *P<0.05 versus CRD.

Figure 4

The antinociceptive effect of ATB-429 is modulated by K_{ATP} channels. Pre-treating healthy rats with the K_{ATP} channel blocker glibenclamide (2.8 µmol/kg i.v.) abrogates the antinociceptive (panel A) and myorelaxant (panel B) effects of ATB-429 (100 mg/kg i.p.). These results are confirmed by quantitative RT-PCR data on spinal c-Fos expression (panel C). Data are mean ± SE of 5 rats. *P<0.05 versus control.

Figure 5

Lack of inflammation after TNBS-induced colitis

Colitis is almost resolved 4 weeks after TNBS administration, as demonstrated by the colonic MPO (panel A) and macroscopic inflammatory score (panel B). CRD alone, mesalamine (100 mg/kg i.p.) and ATB-429 (100 mg/kg i.p.) do not modify these two parameters. Data are mean ± SE of 5 rats.

Figure 6

ATB-429 exerts antinociceptive effects in allodynic rats. Colonic inflammation induced by TNBS causes allodynia and hyperalgesia (panel A) that are completely reversed by

ATB-429 at the dose of 100 mg/kg (panel E), but not by mesalamine administered at the same dose (panel C). However, in contrast to data obtained in healthy rats, TNBS-induced colitis elicits a significant decrease in colorectal compliance (panel B) that is not modified by mesalamine (panel D) or ATB-429 (panel F). Data are mean ± SE of 5 rats. *P<0.05. The antinociceptive effects of ATB-429 are confirmed by RT-PCR analysis of spinal c-Fos mRNA expression (panel G). Data are mean ± SE of 5 rats. *P<0.05 versus control.

Figure 7

ATB-429 increases the plasma concentration of H_2S

In both healthy (panel A; data are mean ± SE of 5 rats. *P<0.05 versus control) and post-colitic (panel B; data are mean ± SE of 5 rats. *P<0.05 versus TNBS) rats, ATB-429 administration induces a significant increase in the plasma concentrations of H_2S.

Figure 8

Colonic CBS and CSE are modulated by inflammation

Colitis induces an overexpression of both CSE (panel A) and CBS (panel 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 ± SE of 5 rats. *P<0.05 versus control.

Figure 9

Induction of colonic pro-inflammatory 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

(panel B) and IL-1β (panel D), while mesalamine (100 mg/kg i.p.) had no effect. Data are mean ± SE of 5 rats. *P<0.05 versus control.

JPET Fast Forward. Published on July 19, 2006 as DOI: 10.1124/jpet.106.106435
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Tables

Table 1.

Gene	Sense primer	Antisense primer
rCBS	ccaggacttggaggtacagc	tcggcactgtgtggtaatgt
rCSE	gtattgaggcaccaacaggt	gttgggtttgtgggtgtttc
rcFOS	gtctggttccttctatgcag	taggtagtgcagctgggagt
rCOX1	cgaggatgtcatcaaggag	tcagtgaggctgtgttaacg
rCOX2	tcaagacagatcagaagcga	tacctgagtgtctttgattg
rTNFa	tgatccgagatgtggaactg	cgagcaggaatgagaagagg
rIL-1β	tgacccatgtgagctgaaag	gggattttgtcgttgcttgt
rcNOS	aacagtggcaacatcaggtcgg	ggtcgatgcacaactgggtgaa
rCGRP	ttggctattgtgcatcgtgt	gtgtccccagaagaccaaga
rTAC1	agcctcagcagttctttgga	agttctgcattgcgcttctt
rTAC2	ggaaggattgctgaaagtgc	gcccataagtcccacaaaga
rGAPDH	atgactctacccacggcaag	atgactctacccacggcaag

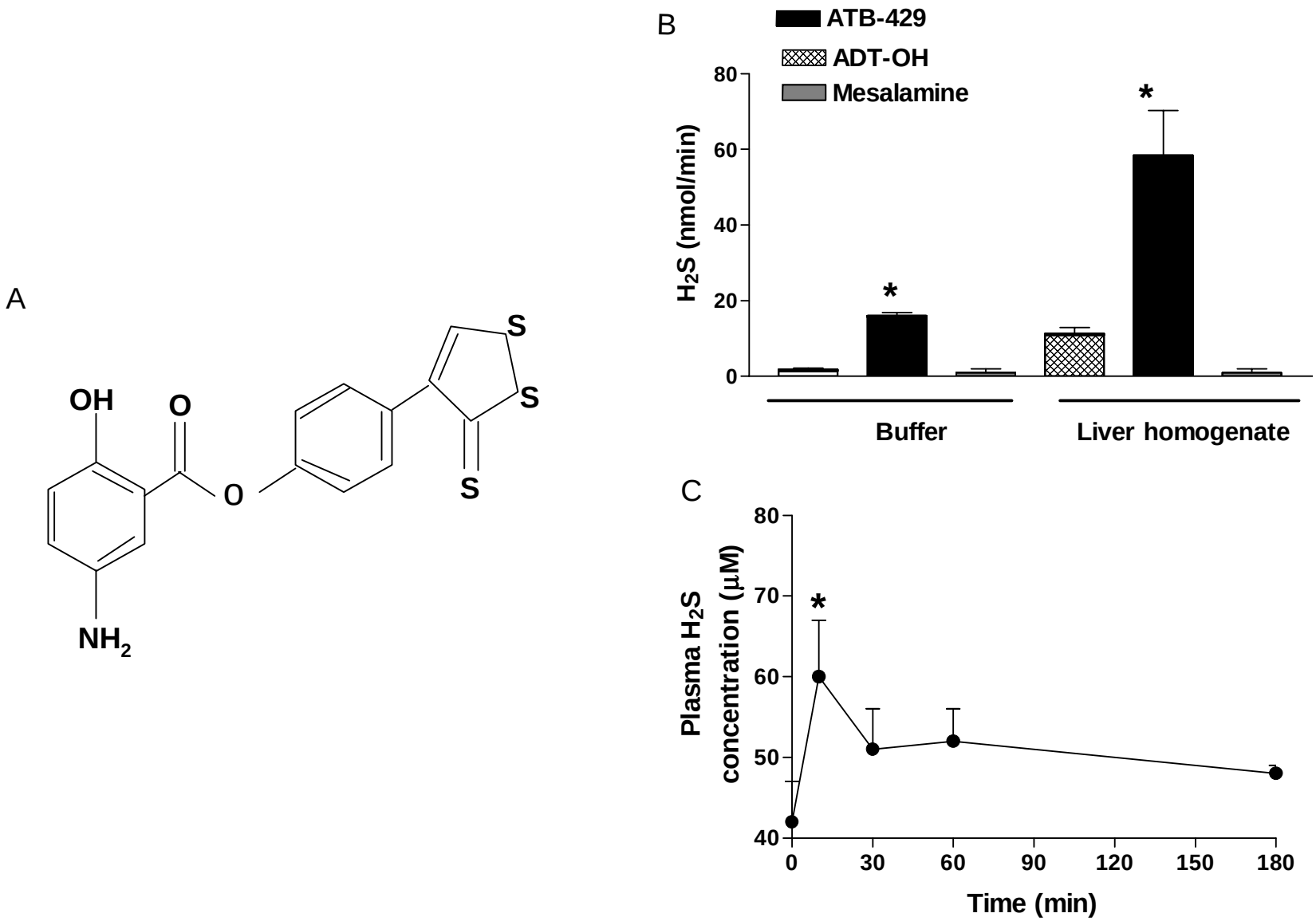
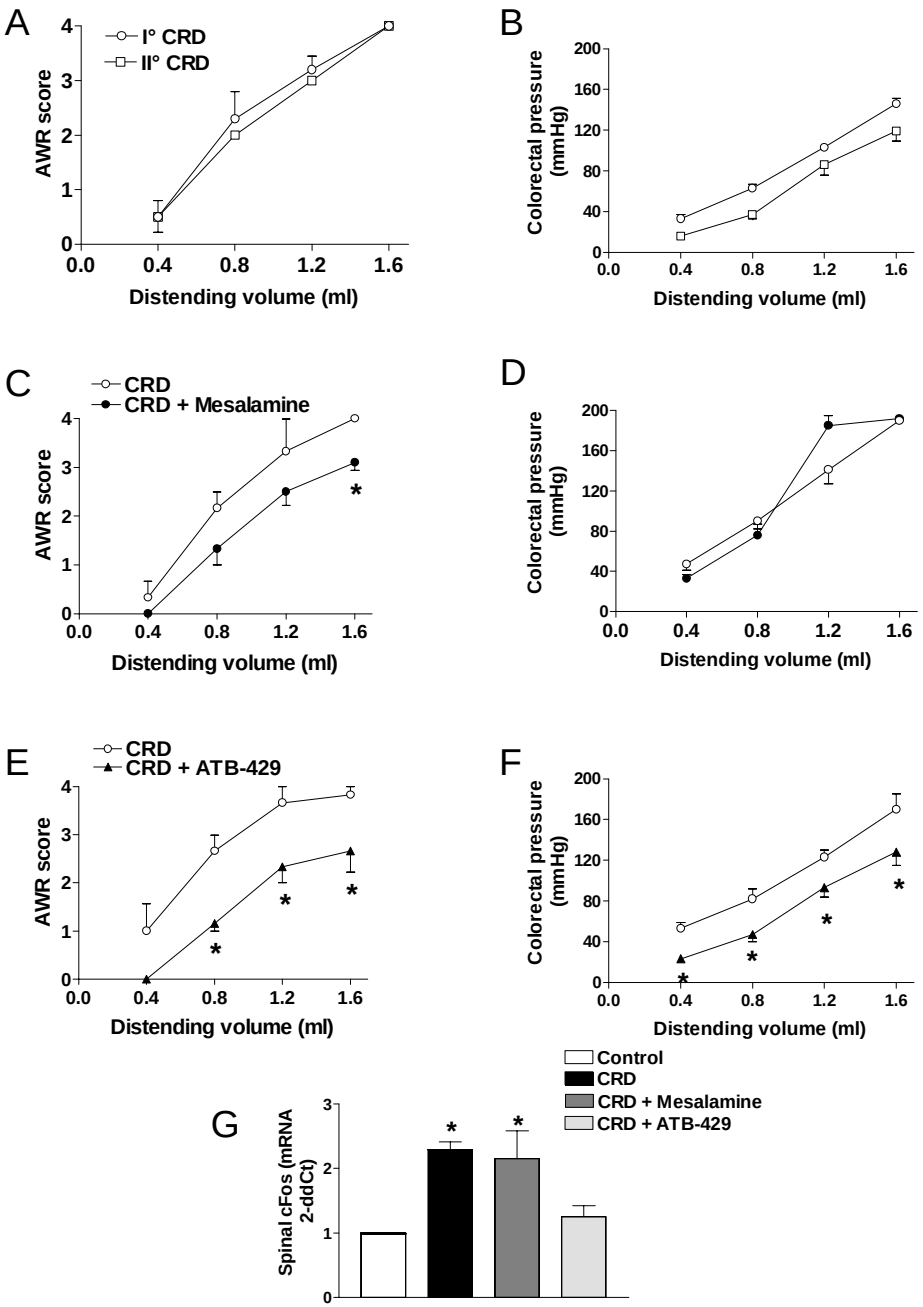


Figure 1



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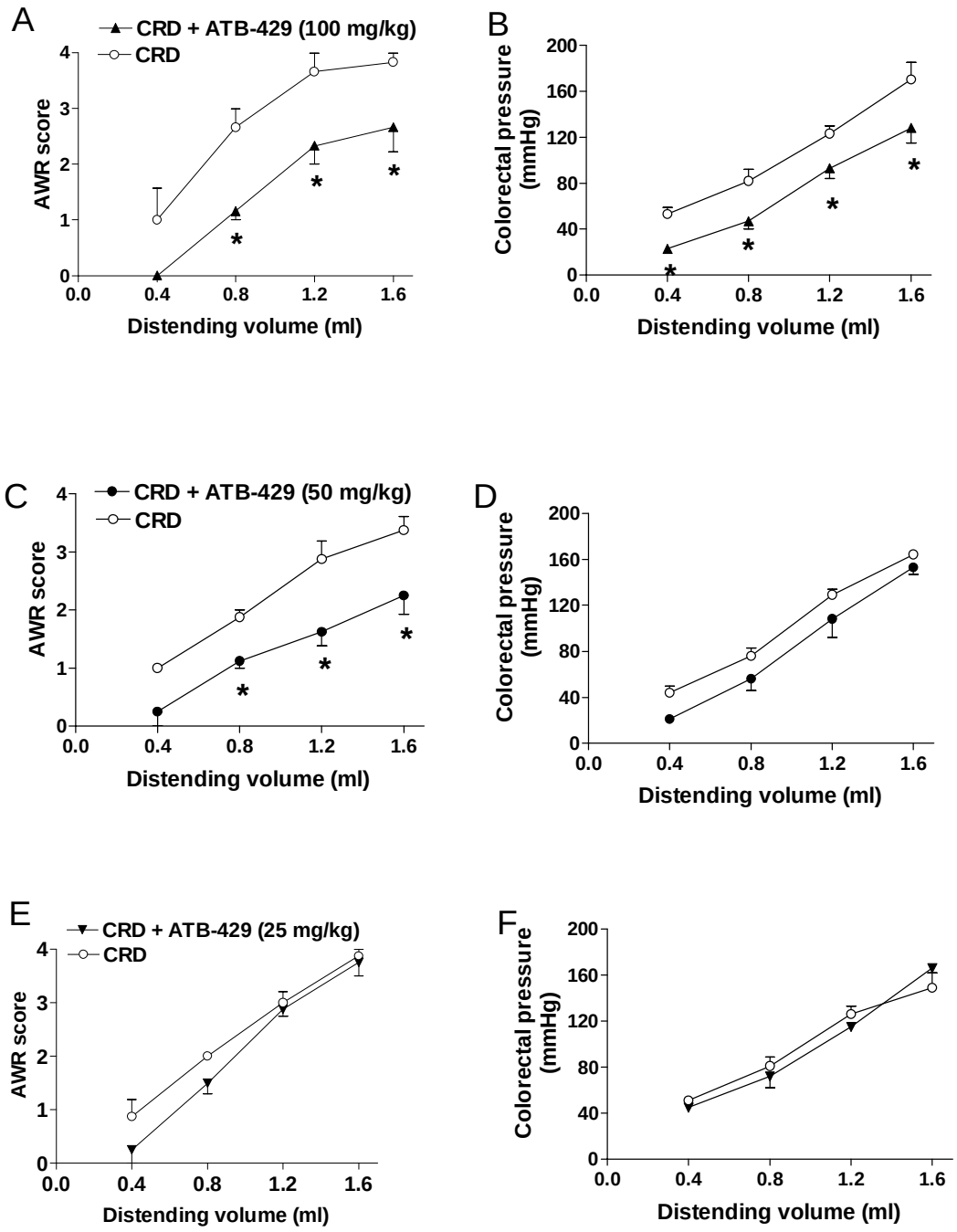

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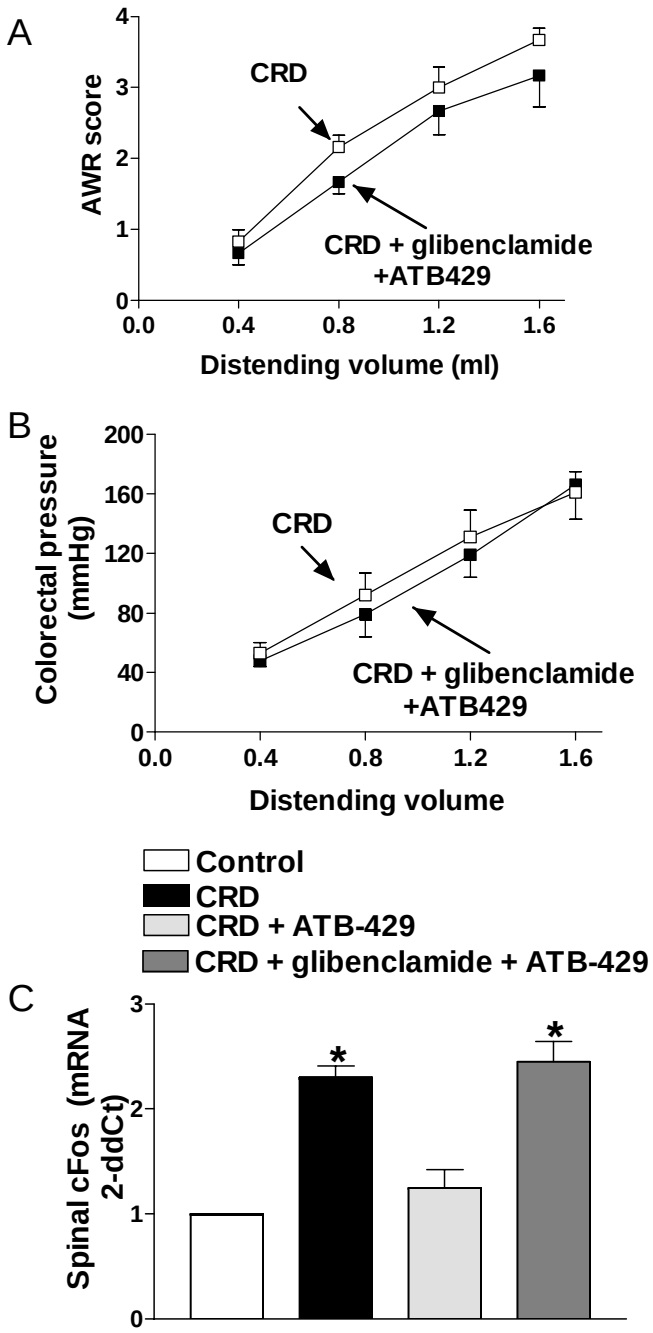

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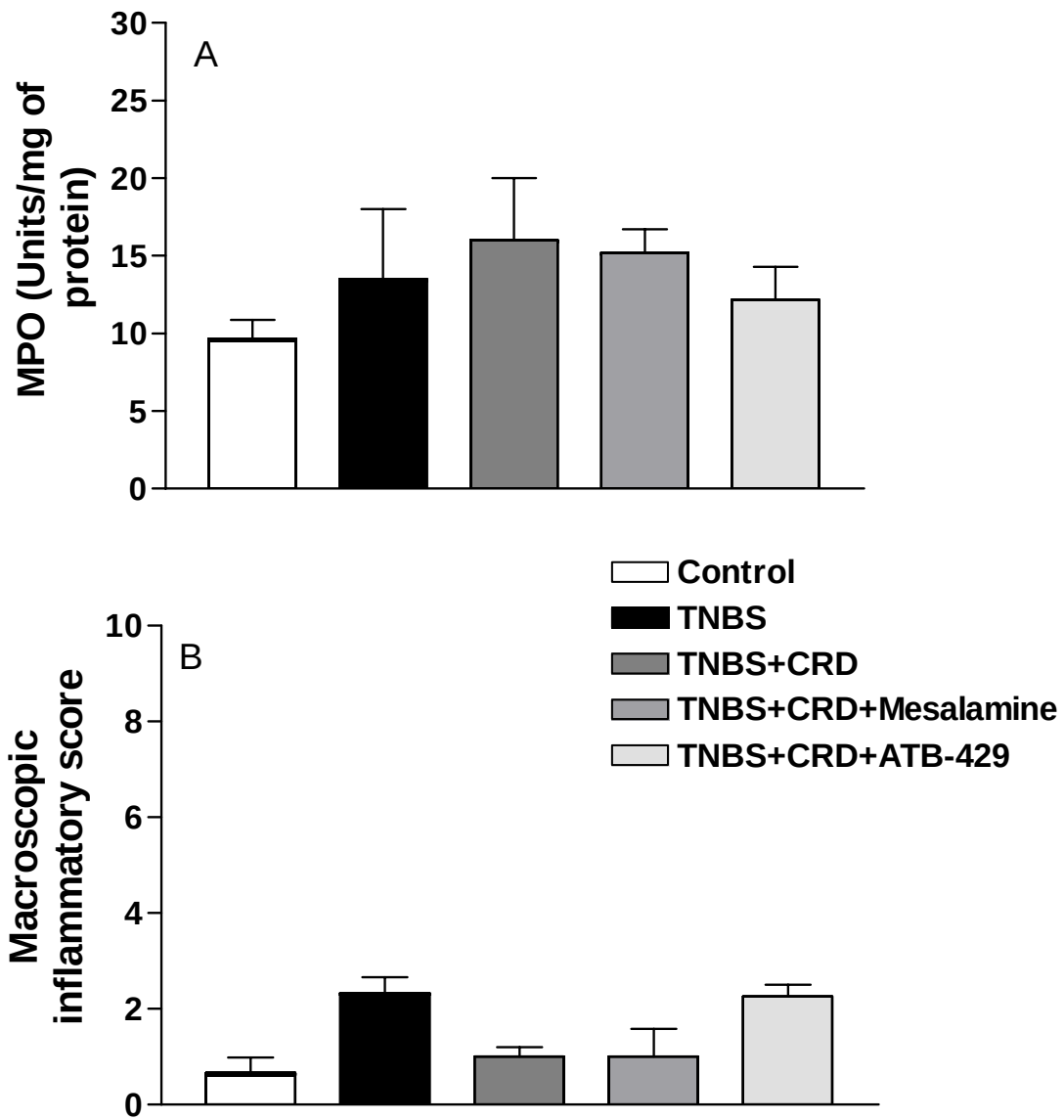

Figure 5

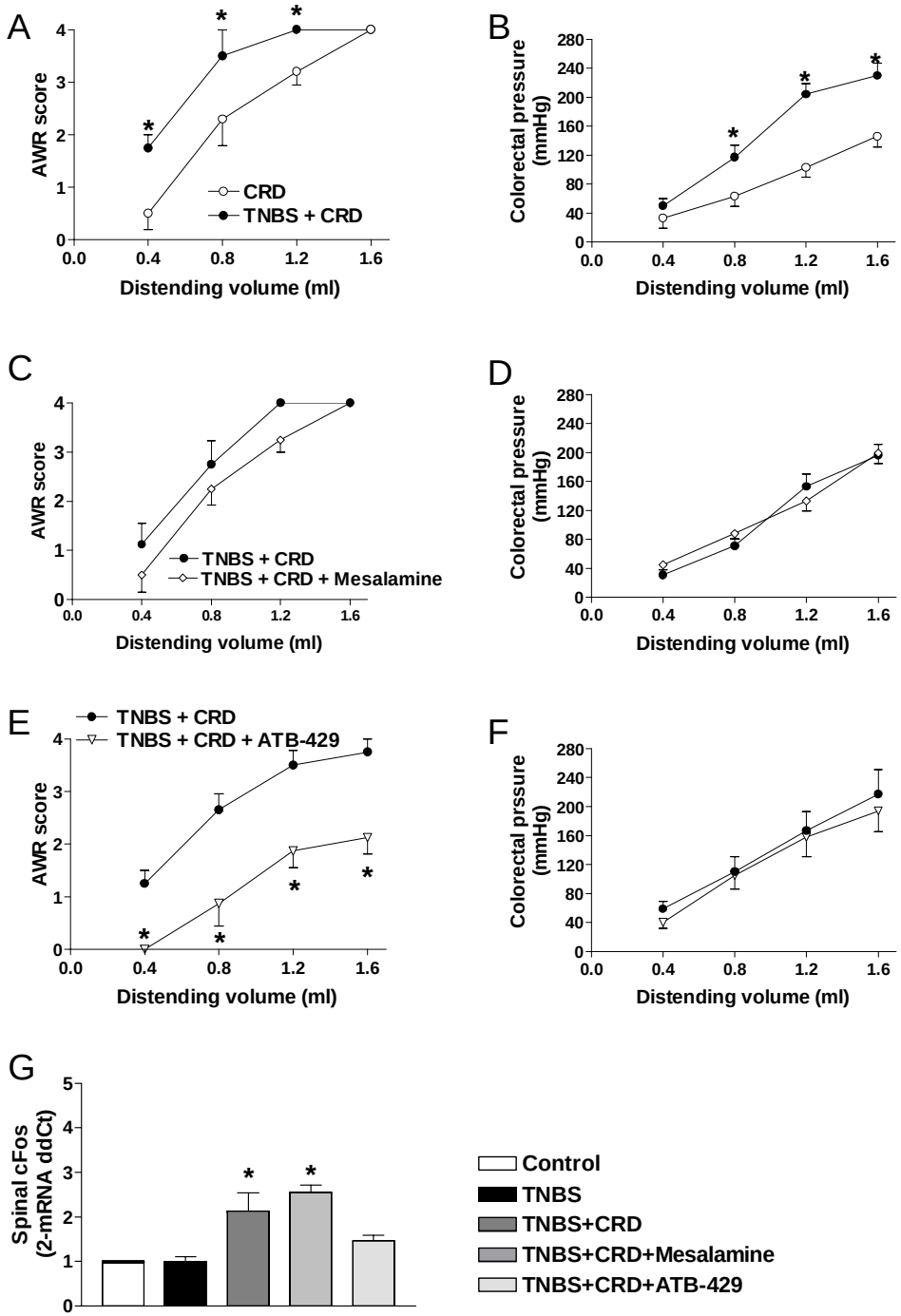

Figure 6

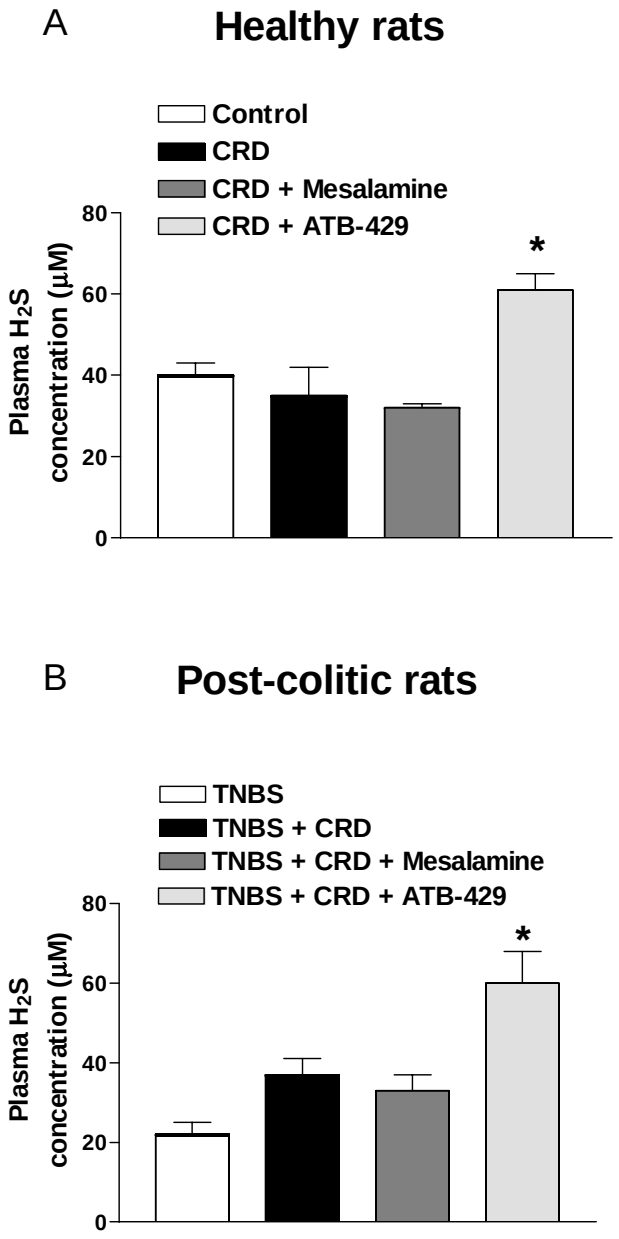

Figure 7

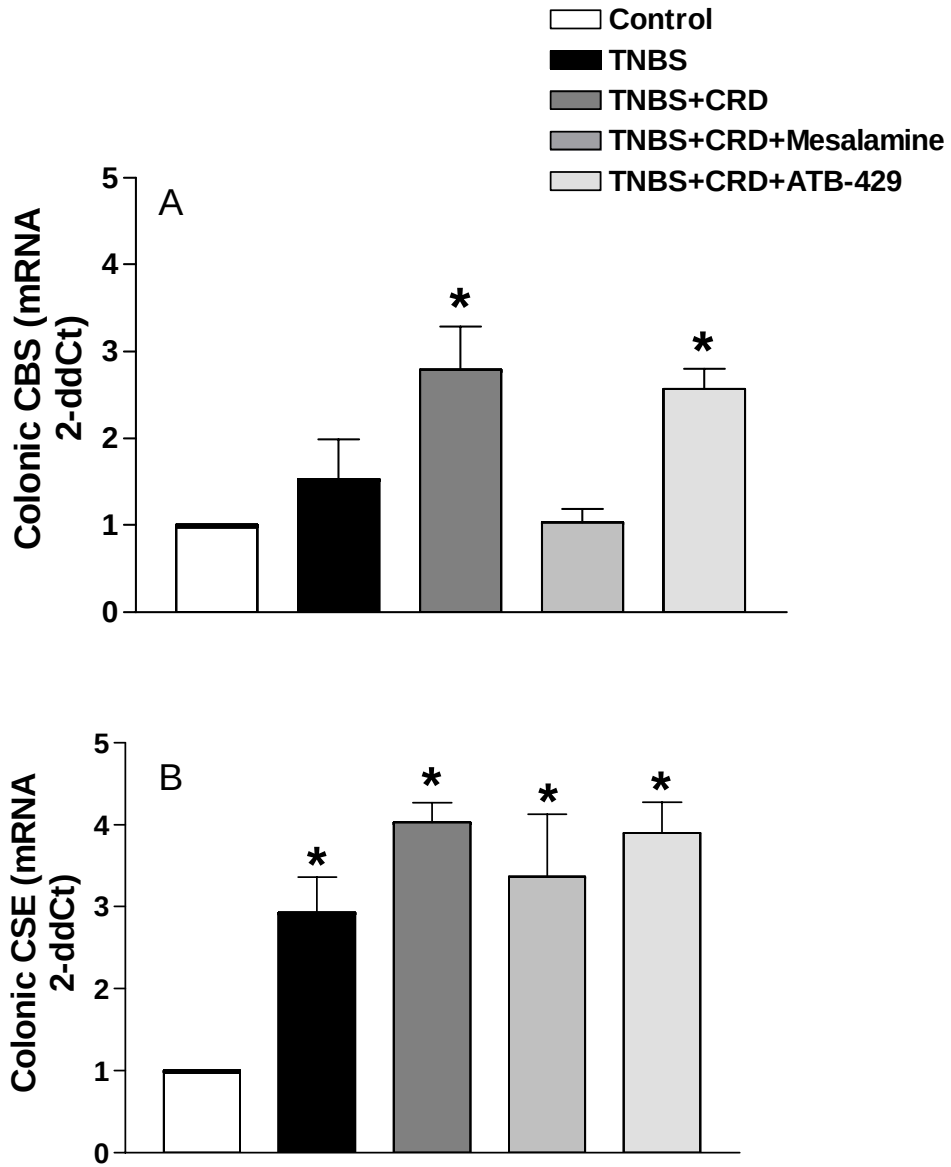

Figure 8

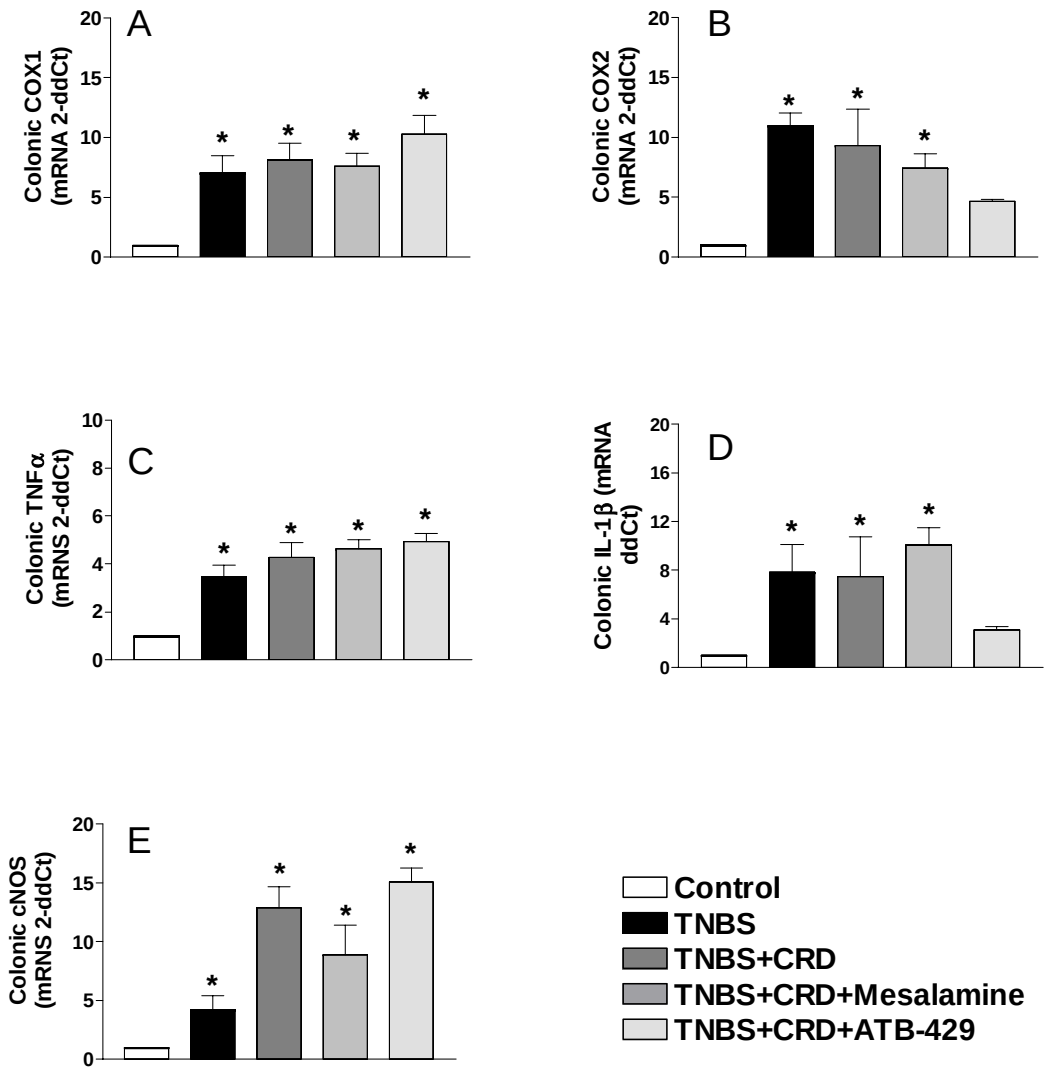

Figure 9