Supplementary Material

Methods

Patients

23 IBS-D patients (18 females and 5 males), age ranging from 28 to 60 years, were recruited from the University of Michigan, Division of Gastroenterology and Hepatology outpatient clinic and primary care clinics. All IBS-D patients met the Rome IV criteria and were symptomatic at the time of the study. Patients had to be free of concomitant diseases such as diabetes and cardiovascular disorders. Celiac disease was excluded by antibodies to anti-tissue transglutaminase or duodenal biopsies. Biopsies of colonic tissue excluded microscopic colitis. Small bowel bacterial overgrowth was excluded with a negative hydrogen breath test. None of the 23 IBS-D patients used NSAIDs, corticosteroids, histamine antagonists, mast cell stabilizers in the last 6 months prior to the study. Twenty-four healthy subjects (15 females and 9 males, ages 36 to 65 years) served as controls. Consents were obtained from all study subjects and the study was approved by the University of Michigan Human Research Protection Program. Healthy controls (HC) were asymptomatic subjects undergoing colonoscopy for colorectal cancer screening. The younger subjects (<50 years old) were referred for screening colonoscopy because of high genetic risk of colon cancer. All subjects had a colonoscopy and 6 mucosal biopsies were obtained from the descending colon of each subject. One biopsy was used for immunohistochemistry to exclude microscopic colitis and 5 biopsies were used to prepare mucosa supernatants.

Animals

All animal procedures were performed in accordance with National Institutes of Health guidelines and with the approval of the University of Michigan Institutional Animal Care & Use Committee. Wistar male rats (200–250 g) were purchased from Charles River Laboratories. C57B16, MC-deficient WBB6F1/J-Kitw/KitW-V/J (W/W^V) and Cox2 mutant B6.129S6(FVB)-Ptgs2tm1.1Fun/J ($Ptgs2^{Y385F}$ mice (6 wk old) were obtained from Jackson Laboratories. All animals were housed 3 per cage in a controlled environment (12-h daylight cycle, lights off at 18:00), with free access to food and water. The animals were euthanized with CO_2 in accordance with the principles and guidelines of the University of Michigan Institutional Animal Care & Use Committee.

Collection of mucosal specimens and assays of supernatants

Human mucosal biopsies and experimental animal colon specimens were collected as previously described,[1] or as specifically indicated in the **Results**. The tissues were rapidly immersed in hard plastic tubes containing 1 mL DPBS media and continuously oxygenated (95% O_2/5% CO_2) at 37°C. After a 30-min incubation, the bathing solution was removed, filtrated, and stored at -80°C. At the end of the experiment, biopsies were weighed. The tissue weight of biopsies obtained from IBS-D patients and healthy controls was 10.9 ± 0.7 mg and 10.3 ± 0.8 mg ($P > 0.05$), respectively.

ELISA assays of PGE2 (#500141, Cayman Chemical, Ann Arbor, MI), histamine (#589651, Cayman Chemical), and tryptase (Pierce Protease Assay Kit, #23263, Thermo Fisher Scientific, Rockford, IL) were performed according to the instructions provided by the manufacturer.

Mast cell reconstitution

Selective reconstitution of mast cells in mast cell–deficient W/W^V mice was conducted according to the method described by Rijnierse.[2] Bone marrow–derived mast cells were obtained from wild-type (C57B16) and Ptgs2^{Y385F} mutant mice. Bone marrow was aseptically flushed from femurs and cultured for 4 wk in RPMI 1640 medium containing 10% FCS, 4 mM l-glutamine, 0.5 μM 2-ME, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mM nonessential amino acids. Recombinant mouse IL-3 (10 ng/mL, #PMC0034, Life Technologies, Rockford, IL) and stem cell factor (SCF/mouse kit ligand; 10 ng/mL; #TP723398, OriGene Technologies, Rockville, MD) were added to the medium to stimulate bone marrow cell development to mature mast cells. Medium was refreshed once a week and fresh IL-3 and stem cell factor were added. Purity of the mast cell population was determined by staining cells with toluidine blue, which indicated that nearly 99% of the viable cells were mast cells after the 4-wk culture. Mast cell–deficient W/W^V mice were injected via the tail vein with 5×10^6 cultured mast cells and the recipients were studied 4–5 weeks later.

Colorectal distension and electromyography recording

Measurement of visceral hypersensitivity in animals was based on brain stem reflexes, which have been described as pseudo affective responses.[3] The visceromotor responses (VMR) were recorded by quantifying reflex contractions of the abdominal musculature induced by colorectal distension (CRD) The animals were anesthetized with a mixture of xylazine and ketamine (13 and 87 mg/kg body wt, respectively) as described previously.[4] Electromyography electrodes were implanted into the external oblique pelvic muscles 2–3 days before the beginning of the

experimental procedures. The skin was sutured over the strain gauge, and the lead wires were looped around the animal's flank and secured with a single suture inhman

the skin. During the experiment, the strain gauge was connected by way of a shielded cable to a chart recorder to monitor the number of abdominal muscle contractions. A latex balloon 3 cm long for rats and 0.7 mm for mice (Edwards Lifesciences, Irvine, CA) lubricated with glycerol was inserted into the colon through the rectum 30 min before CRD was initiated. Biopsy supernatants from healthy controls or IBS-D patients (1 mL for rats and 0.15 mL for mice) were administrated intracolonically, proximal to the rectum (75 mm in rats and 30 mm in mice). At set times after the sample administration (0, 1.5, 3, 6, 10, and 24 h; 3, 7, 14, and 21 days), a series of rectal distensions were performed to generate a pressure-response curve. The effects of supernatants from individual patients were studied in separate experiments except in a few cases, where noted when supernatants from 2 or more IBS-D patients were combined to provide sufficient sample volume to enable more detailed multiple experiments to be performed at different time points. The responses were considered stable if there was less than 20% variability between two consecutive trials of CRD. The results of electromyography were amplified and filtered (A-M Systems, Sequim, WA), digitized, and integrated by using the SPIKE2/CED 1401 data-acquisition interface. Spike bursts higher than 0.3 mV were regarded as significant and were, therefore, used to estimate the response. The increase in the area under the curve of EMG amplitude during CRD over the baseline period before CRD was recorded as the response.

Measurement of colorectal compliance

As described above, the rats were equipped with a 3-cm latex balloon inserted into the distal colon. In this set of experiments, the barostat system simultaneously inflated the balloon to a

predetermined pressure and measured the volume changes in the balloon. The pressure-dependent changes in response to volume distension during isotonic CRD were recorded as a measure of colorectal compliance. The distension paradigms generated by the barostat were achieved by generating pulse patterns on an analog output channel. The CRD protocol consisted of 4 phasic distensions at 20, 40, 60, and 80 mm Hg. Each distension lasted for 30 s, with a 5-min interval between consecutive distensions.

Chemicals

To assess the effects of mast cell degranulation, mast cell degranulation inhibitor cromolyn sodium (30 mg/kg, intraperitoneal [IP], Sigma-Aldrich, St Louis, MO);[5] H1 receptor inhibitor and mast cell degranulation inhibitor olopatadine HCl (1 mg/kg, IP, R&D Systems, Minneapolis, MN);[6] serine protease inhibitor nafamostat mesylate (1 mg/kg, R&D Systems)[7] were injected intraperitoneally 30 min before intracolonic administration of healthy controls or IBS-D colonic biopsy supernatant. PAR2 agonist SLIGKV-NH2 or its reverse sequence peptide LSIGKV-NH2 (0.5 mg/ml, R&D Systems, Minneapolis, MN, USA), histamine (30 µg/ml, R&D Systems), and PGE2 (30 µg/ml, Cayman Chemical) were administrated intracolonically. Celecoxib, a specific Cox2 inhibitor (7 mg/kg, IP, R&D Systems),[8] PF04418948, a specific EP2 receptor antagonist (10 mg/kg, IP, R&D systems),[9] and EP2 receptor blocking peptide SLRTQDATQTSCSTQSDASKQADL (10 µg/kg, IP, Cayman Chemical), were administered after completion of VMR–CRD test in response to intracolonic administration of supernatants and VMR to CRD were repeated 30 min later. ABP1/AOC1/diamine oxidase (1 mU, R&D Systems) was mixed with IBS-D colonic biopsy supernatant 30 min before intracolonic administration.

Isolation of mast cells from rat colon

Collagenase (type I), dipase (type I-S), Triton X-100, trypan blue, Percoll, FCS, RPMI 1640, gentamicin, and penicillin/streptomycin were obtained from Sigma-Aldrich. Percoll stock solution was prepared by mixing the commercial Percoll solution and 10× HEPES buffer plus distilled water to obtain an osmolality of 285 mOsm/kg H_2O and a final density of 1.123 g/mL. The desired densities of Percoll were achieved by mixing the Percoll stock solution with RPMI 1640 media.

Tissue was obtained from the colon of Wistar rats (200–250 g). The digestion process (30 min at 37°C on a shaker) was first performed with a low concentration of enzymes (2 digests at 200 U/g in 10 mL final volume). This was followed by digestion with a high concentration of enzymes (2 digests at 2000 U/g in 10 mL final volume). Each digest was followed by washing the enzyme-treated tissue over a 100-micron nylon mesh filter to collect freed cells. After the digestion procedure, the freed cells were pelleted, pooled, resuspended in RPMI 1640 media, and layered over a single density Percoll gradient (1.041 g/ml) and centrifuged (500 *g*, 20 min). The resulting top cell layer (mononuclear and damaged cells) was removed. The mast cell–enriched pellet was washed in RPMI 1640 media, resuspended to a concentration of 1×10^6 cells/ml in mast cell culture medium, and transferred to a 24-well plate (0.5 mL/well) for an equilibration period (up to 24 h at 37°C). After the equilibration period, the cell suspension was removed from the plate, layered over a double density Percoll gradient (1.08 g/mL layered over 1.123 g/mL) and centrifuged (500 *g*, 20 min).

The viability of the mast cells was determined by staining with trypan blue and counting on a hemocytometer. The purity of the mast cell population was determined by staining cells with toluidine blue, which indicated that nearly 90% of the viable cells were mast cells.

Mediator release from purified colonic mast cells

Purified colonic mast cells ($2–5 \times 10^4$ mast cells/tube) suspended in 3 mL RPMI 1640 media were stimulated with PAR2 agonist (10 μg/mL) or histamine (10 μM) for 0.5 to 5 h at 37°C. Cell pellets and supernatants were harvested, stored at −70°C and assayed in duplicate for PGE2 (supernatants diluted 1:10) according to manufacturers' instructions.

Immunohistochemistry

For immunohistochemical staining, colonic biopsies were cut with a cryostat (5 μm: Leica CM1800, Leica Microsystems, Wetzlar, Germany) and permeabilized in PBS with 0.3% Triton X-100 for 10 min. After blocking with 10% goat serum (Vector Laboratories, Burlingame, CA) in PBS with 0.3% Triton X-100, sections were incubated with the following antibodies: mouse monoclonal anti-COX2 (#160112, 1:500, Cayman Chemical) or rabbit polyclonal anti-COX2 (sc-7951, 1:200 Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal anti-mast cell tryptase (sc-17039, 1:500, Santa Cruz Biotechnology) incubated for 18 h at 4°C. Cy3 and FITC conjugated donkey anti-rabbit and anti-mouse antibodies (1:500; Jackson ImmunoResearch, West Grove, PA) were used as secondary antibodies to detect COX2 and mast cell tryptase. Incubation for 2 h was performed at room temperature. The specificity of anti-mast cell-tryptase and anti-COX2 antibodies were verified in mast cell (W/W^V) and $Ptgs2^{Y385F}$ mutant mice, by showing the lack of particular immunoreactive staining for mast cell tryptase and COX2 in these

mice respectively. The slides were examined using the ×20 objective with a BX51 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with FITC (U-MNIB2) and CY3 (U-MWIG2) filters, and a digital camera. The images were analyzed in bright field light and in fluorescent light using filter combinations, which enabled separate visualization of multiple fluorophores.

Western blot analysis

Colonic biopsies were homogenized in ice-cold lysis buffer. The homogenate was centrifuged at 14 000 *g* for 10 min. Protein samples were run on Ready Gel 10% Tris-HCl (Bio-Rad, Hercules, CA) for 1.5 h at 80 V. Proteins were transferred to PVDF membranes for 1.5 h at 80 V. The membranes were blocked with TBS supplemented with 0.5% dry milk (Bio-Rad) for 1 h at room temperature, probed with primary antibodies against COX2 (mouse monoclonal #160112, clone CX229, Cayman Chemical) and GAPDH antibody (SC-25778, Santa Cruz Biotechnology) at 1:2000 dilution at 4°C overnight, and then washed in TBS for 1 h. The membranes were probed with corresponding HRP-conjugated secondary antibodies at 1:2500 dilution for 1 h at room temperature and the bands were visualized by electrochemiluminescence (PerkinElmer, Waltham, MA). Signals were quantified using ImageJ (National Institutes of Health, Bethesda, MD) and normalized to controls.

Intrathecal ganglionic administration of EP2 receptor siRNAs

Male Wistar rats weighing 200–250 g were used. After shaving the fur over the lower back, rats were given isoflurane anesthesia (2.5%) and placed over a small cylinder to elevate the lumbar region. Intrathecal injection was performed according to a technique described by Mestre et al.[10]

Injections were performed by holding the rat securely in one hand by the pelvic girdle and inserting a 25 gauge 1″ needle connected to a 25-1×L Hamilton syringe into the tissue between the dorsal aspects of L5 and L6, perpendicular to the vertebral column. When the needle entered the subarachnoidal space a sudden lateral movement of the tail was observed, indicating successful puncture. 3 μL of EP2 siRNA (sc-45910, Santa Cruz Biotechnology) or their mismatched controls (sc-37007, Santa Cruz Biotechnology) were mixed with 10 μL of iFect (Neuromics, Edina, MN) were injected in a final volume of 10 μL. The syringe was then held in position for a few seconds and slowly removed to avoid any outflow of the drug.

Reverse transcriptase–PCR studies

Total RNA extraction. Gene expressions of COX1 and COX2 were measured by RT-PCR. Human colonic biopsy tissue or rat isolated primary DRG neurons were lysed in reverse transcriptase buffer. Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA). cDNAs were synthesized with reverse transcriptase (Promega, Madison, WI) using 5 μg of total RNAs; the resultant cDNAs were used for PCR. PCR was performed with Taq DNA polymerase (Promega) through variable numbers of cycles; denaturation (1 min at 94°C), annealing (2 min at 50°C), and extension (3 min at 72°C), with a final extension period of 10 min at 72°C. Primer sets targeting various mRNAs are listed in Table 1. The housekeeping gene GAPDH served as an internal control. The PCR products were loaded in 1.2% Tris/borate/EDTA–buffered agarose gel and bands were visualized after gel electrophoresis by ethidium bromide staining and UV light illumination. The resulting bands were scanned with an EPSON 2400 and analyzed using ImageJ.

Retrograde tracing of DRG neurons

Wistar male rats were deeply anesthetized with a mixture of isoflurane in air, as described previously.[4] After a laparotomy, the retrograde tracer DiI was applied to the distal colon. To confine the dye to the application site, CM-DiI crystals (Thermo Fisher Scientific) were embedded in a fast-hardening epoxy resin that was allowed to harden for about 5 min. The wound was closed with nylon sutures (4-0). The animals were allowed to recover for 12–14 days before being killed for the harvesting of L6–S2 ganglia.

Statistical analysis

Differences of quantified data between groups were compared using one-way ANOVA followed by a post hoc Bonferroni test or a Student *t* test if only two groups were applied. The visceromotor response (VMR) was compared using two-way repeated-measures ANOVA, using baseline values as a covariate and two main factors (i.e., distension level as the repeated factor and group as the independent factor). Results are expressed as means ± SEM. P value < 0.05 was considered statistically significant.

References

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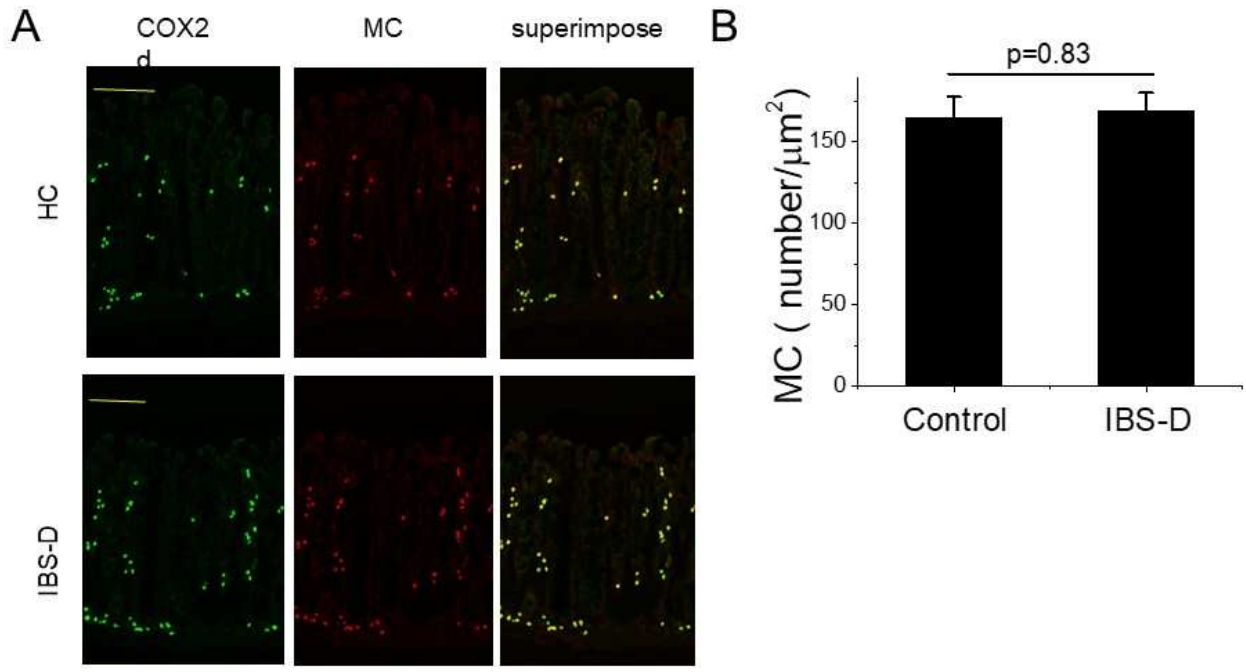
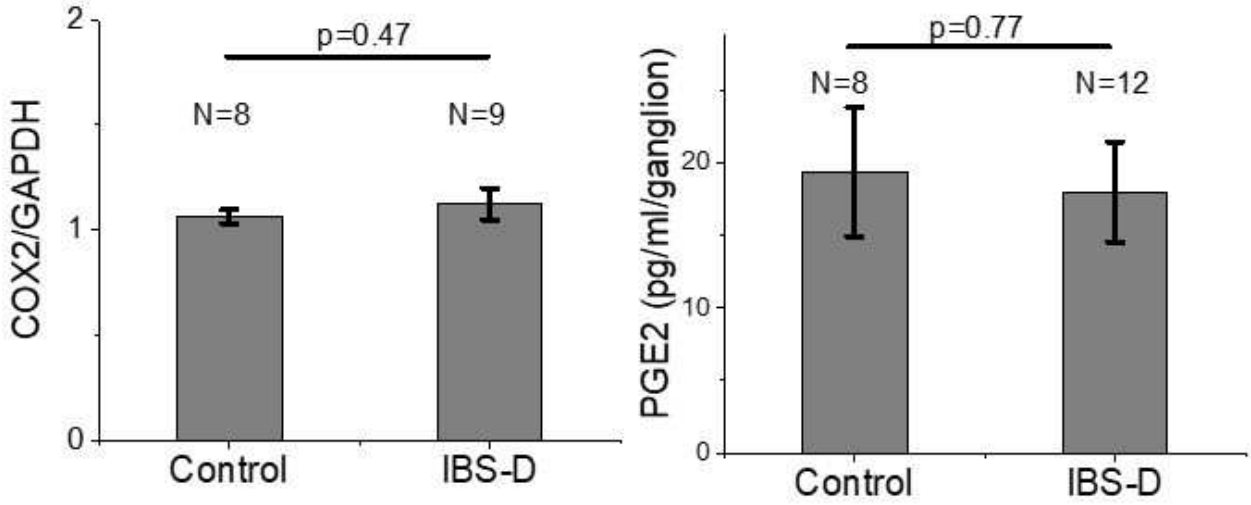
Table 1 RT-PCR primers

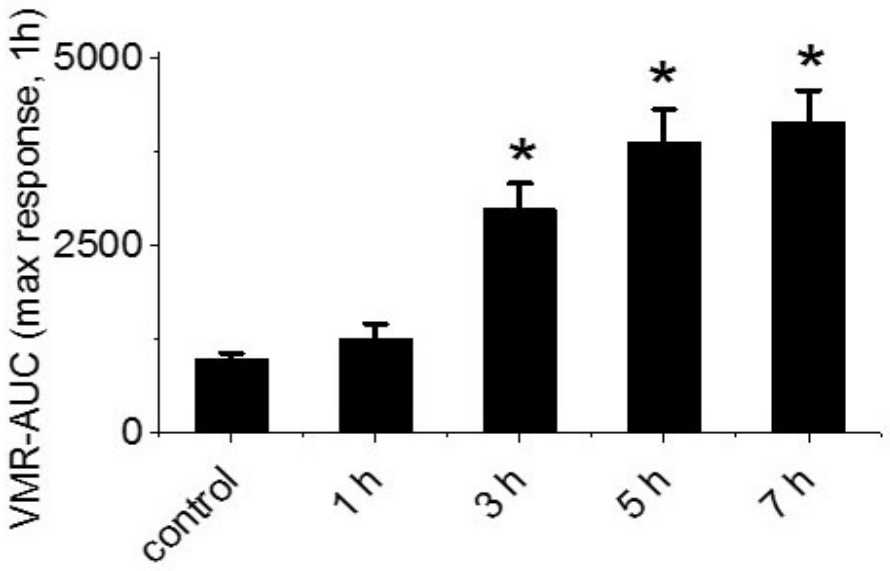
Gene		Primer
Cox1	Sense	5′-CCACCTACAACTCAGCACATG-3′
Human	Antisense	5′-CCGCAGTTGATACTGACGCTC-3′
Cox2	Sense	5′-CCGCAGTTGATACTGACGCTC-3′
Human	Antisense	5′- GCGCAGTTTACGCTGTCTAGC-3′
Cox 1	Sense	5′-GCAGTCCTTCAATGAATACCGA-3′
Rat	Antisense	5′-CCATATAGCTCCTCCAACTCAG-3′
Cox2	Sense	5′-AGACAGATCAGAAGCGAGGA-3′
Rat	Antisense	5′-TCTCCACCGATGAGAT-3′
IL1β	Sense	5′-GTGCTGTCTGACCCATGT-3′
Rat	Antisense	5′-TTGTCGTTGCTTGTCTCTCC-3′
INFγ	Sense	5′-CGCACCTGATCACTAACTTCT-3′
Rat	Antisense	5′-GAGTTCATTGACAGCTTTGTGC-3′
TNFα	Sense	5′-AGACCCTCACACTCAGATCA-3′
Rat	Antisense	5′-GTCTTTGAGATCCATGCCATTG-3′
Ep2	Sense	5′-TCGCCGGAGAGGAGAAAGGAC-3′
Rat	Antisense	5′-TAAGGATGACAAAACCCAAGGAT-3′
GAPDH	Sense	5′-CACCACCATGGAGAAGGCTGG-3′
	Antisense	5′-GGGCACTTTAACGGTGTTCCC-3′



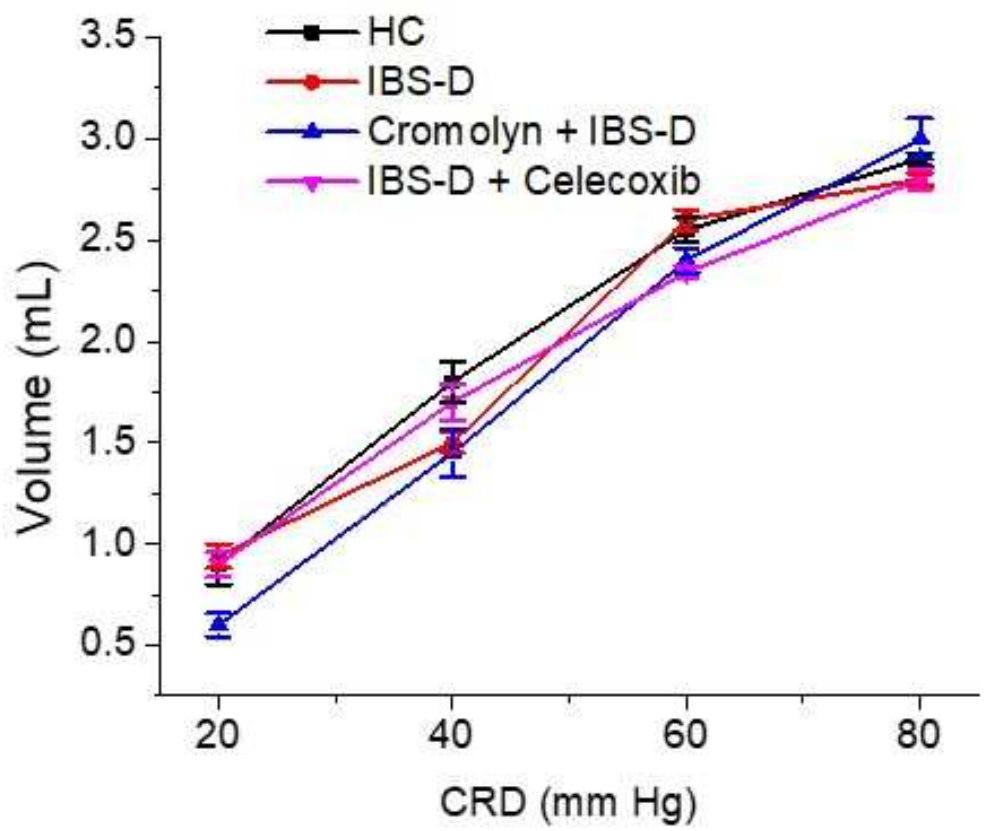
Supplementary Figure 1. Colon mucosa tryptase and COX2 expression in response to intracolonic perfusion of mucosal supernatant obtained from healthy controls (HC) and IBS-D patients. (**A**) Expression of mast cell tryptase and COX2 in rat colonic tissue. (**B**) Bar graph shows tryptase immunoreactivity cell counts in rat colonic mucosa (n = 6 each group).



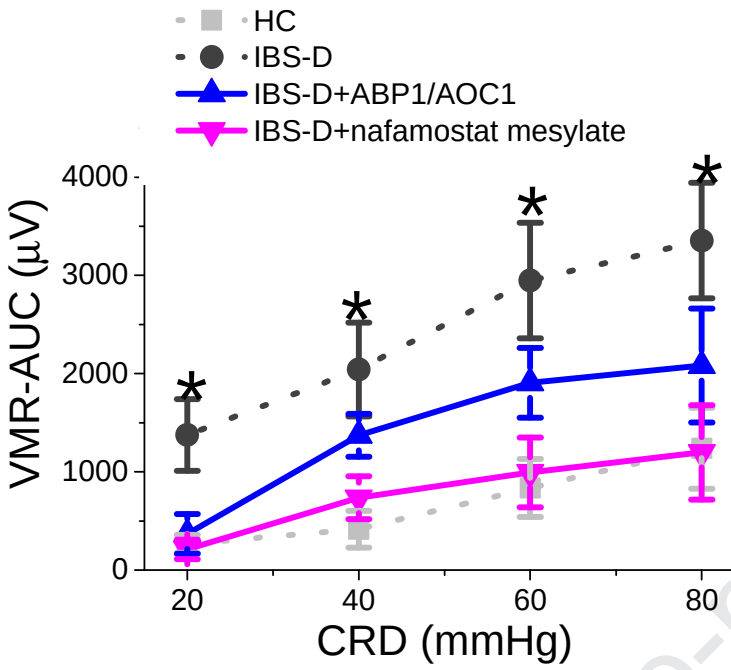
Supplementary Figure 2. Summary bar graph showing the levels of COX2 (left panel) and the release of PGE2 (right panel) from L6-S2 ganglia in response to intracolonic administration of biopsy supernatants from HC or IBS-D patients measured 6 h after administration.



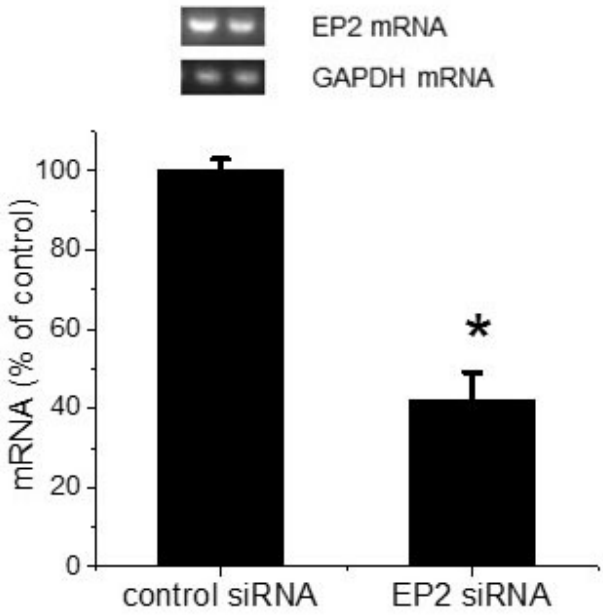
Supplementary Figure 3. Summary bar graph shows maximal amplitude of VMR in response to 80 mm Hg CRD before (control, PBS) and at various time intervals after intracolonic administration of IBS-D mucosal supernatant (n = 3, *P < 0.05 from control; two-way ANOVA followed with Bonferroni post-hoc test).



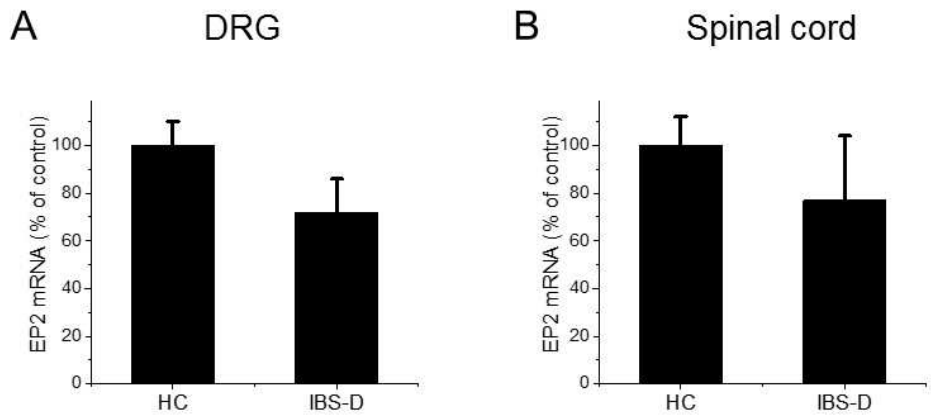
Supplementary Figure 4. Effects of HC or IBS-D supernatant, with or without cromolyn sodium (30 mg/kg, IP, 1 h before administration of IBS supernatant) or celecoxib (3 μmol/kg, IP) on the pressure–volume relationship during CRD in rats. Rectal compliance in response to a range of CRD was determined 3 h after administration of colonic biopsy supernatant (HC or IBS-D). Stimulations were 20, 40, 60, and 80 mm Hg. Mean ± SEM. *n* = 3 rats in each group. No significant difference between groups. ANOVA followed with Bonferroni post-hoc test.



Supplementary Figure 5. Mucosal supernatant of IBS-D patients (black trace) induced VH in rats, compared with HC mucosal supernatant (grey trace), an effect that was partially or completely prevented by co-administration of IBS-D colonic biopsy supernatant with the serine protease inhibitor nafamostat mesylate (red trace, 100 mM, $n = 4$) or ABP1/AOC1/diamine oxidase (blue trace, 1 mU, $n = 4$). *$P < 0.05$ from HC.



Supplementary Figure 6. Summary bar graph shows that intrathecal treatment of rats with EP2 siRNA significantly reduced the levels of EP2 mRNA in L6–S2 DRG, compared to rats that received intrathecal treatment with control siRNA ($n = 5$, $^*P < 0.05$ compared with controls, Student *t* test).



Supplementary Figure 7. Summary bar graph showing visceral hypersensitivity induced by intracolonic administration of IBS-D supernatant was not associated with upregulation of EP2 receptor in the L6–S2 DRG (**A**) or the spinal cord (**B**). GADH was used as a loading control (n = 5 per group, $P > 0.05$ compared with HC, Student t test).