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Protease-Activated Receptor 2, Dipeptidyl Peptidase I, and Proteases Mediate *Clostridium difficile* Toxin A Enteritis

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Abstract

Background & Aims—We studied the role of protease-activated receptor 2 (PAR₂) and its activating enzymes, trypsins and tryptase, in *Clostridium difficile* toxin A (TxA)-induced enteritis.

Methods—We injected TxA into ileal loops in PAR₂ or dipeptidyl peptidase I (DPPI) knockout mice or in wild-type mice pretreated with trypsin inhibitors (FUT-175 or MPI-0442352) or soybean trypsin inhibitor. We examined the effect of TxA on expression and activity of PAR₂ and trypsin IV messenger RNA in the ileum and cultured colonocytes. We injected activating peptide (AP), trypsins, tryptase, and p23 in wild-type mice, some pretreated with the neurokinin 1 receptor antagonist SR140333.

Results—TxA increased fluid secretion, myeloperoxidase activity in fluid and tissue, and histologic damage. PAR₂ deletion decreased TxA-induced ileitis, reduced luminal fluid secretion by 20%, decreased tissue and fluid myeloperoxidase by 50%, and diminished epithelial damage, edema, and neutrophil infiltration. DPPI deletion reduced secretion by 20% and fluid myeloperoxidase by 55%. In wild-type mice, FUT-175 or MPI-0442352 inhibited secretion by 24%–28% and tissue and fluid myeloperoxidase by 31%–71%. Soybean trypsin inhibitor reduced secretion to background levels and tissue myeloperoxidase by up to 50%. TxA increased expression of PAR₂ and trypsin IV in enterocytes and colonocytes and caused a 2-fold increase in Ca²⁺ responses to PAR₂ AP. AP, tryptase, and trypsin isozymes (trypsin I/II, trypsin IV, p23) caused ileitis. SR140333 prevented AP-induced ileitis.

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Conclusions—PAR₂ and its activators are proinflammatory in TxA-induced enteritis. TxA stimulates existing PAR₂ and up-regulates PAR₂ and activating proteases, and PAR₂ causes inflammation by neurogenic mechanisms.

Protease-activated receptor 2 (PAR₂) belongs to a family of G protein-coupled receptors that are activated by proteolytic cleavage within their extracellular N-terminal domain.^{1,2} PAR₂ is highly expressed in the gastrointestinal tract, where it is present on enterocytes, myocytes, myenteric and submucosal neurons, and extrinsic sensory nerves.²⁻⁴ Proteases that activate PAR₂ include enzymes from inflammatory cells (eg, mast cell tryptase, neutrophil proteinase 3) and epithelial tissues (pancreatic and extrapancreatic trypsins, kallikreins), coagulation factors (VIIa, Xa), and proteases from dust mites and bacteria.^{2,3,5} Trypsins and tryptase are prominent agonists of PAR₂ in the gastrointestinal tract. Pancreatic trypsin in the intestinal lumen may activate PAR₂ at the apical membrane of enterocytes.⁵ However, intestinal epithelial cells express trypsinogen IV, which is activated by enteropeptidase to form trypsin IV, an isozyme that also activates PAR₂.⁶ Tryptase released from mast cells can activate PAR₂ on myocytes, neurons, and epithelial cells.^{3,7} Mast cells require the papain-like cysteine protease dipeptidyl peptidase I (DPPI) for full expression of active tryptase,⁸ and high levels of DPPI are constitutively expressed in the small and large intestine of the mouse, emphasizing its potential role in intestinal inflammation.⁹ Mice lacking DPPI exhibit a 75% reduction in the normal tryptase levels in mast cells and show increased survival from sepsis, which is directly due to the lack of DPPI in mast cells.^{8,10} Proteases that activate PAR₂ are released and activated during inflammation and injury, and PAR₂ controls responses to these insults, with effects on gastrointestinal secretion, motility, inflammation, and pain.^{1,3,5,11-13}

We investigated the role of PAR₂ and proteases that activate PAR₂ in enteritis induced by toxin A (TxA) from *Clostridium difficile*. TxA-induced enteritis in rodents parallels the human infection, a common iatrogenic illness in patients receiving antibiotics. Despite its prevalence, nothing is known about the role of PAR₂ and proteases in TxA-induced enteritis. However, TxA and PAR₂ agonists induce intestinal inflammation by similar mechanisms involving many of the same cell types, suggesting a major role for PAR₂ in TxA-induced enteritis. Enterocytes, intrinsic and extrinsic neurons, and inflammatory cells of the lamina propria participate in the host response to TxA in rodents.¹⁴ TxA induces disruption of tight junctions of enterocytes and compromises the integrity of the epithelial layer.^{15,16} Degranulation of mast cells causes release of calcitonin gene-related peptide and substance P, which activate enteric neurons to increase intestinal secretion, leading to diarrhea.¹⁴ Cytokines, such as interleukin 1 and tumor necrosis factor α , recruit neutrophils, which cause acute inflammation.^{14,16} Similarly to TxA, PAR₂ agonists cause redistribution of tight junctional proteins and increased paracellular permeability of intestinal epithelial cells,⁷ with secretion of prostaglandin E₂.⁵ PAR₂ agonists also cause fluid and electrolyte secretion in the intestine and promote inflammation of the intestine and other tissues by neurogenic mechanisms involving calcitonin gene-related peptide and substance P.^{1,12,17} In view of the similar proinflammatory effects of TxA and PAR₂ agonists in the intestine, and because animals deficient in mast cells or receiving mast cell stabilizers have diminished inflammation in response to TxA,^{18,19} we hypothesized that PAR₂ and PAR₂-activating proteases would have a proinflammatory role in this model.

To test this hypothesis, we examined the role of PAR₂ and the enzymes that activate it, tryptase and trypsin, in TxA-induced enteritis, using both a genetic and a pharmacologic approach. We assessed inflammation by measuring intestinal secretion, measuring myeloperoxidase (MPO) activity in tissue and luminal fluid, and histologic assessment of tissues. Our aims were to (1) investigate the effect of deletion of PAR₂ or the mast cell-processing enzyme DPPI on TxA-induced ileitis, (2) determine the effects of the tryptase inhibitors nafamostat mesilate (FUT-175) and MPI-0442352²⁰ and of soybean trypsin inhibitor (SBTI) on TxA-induced inflammation, (3) evaluate the effects of TxA on expression of PAR₂ and of PAR₂-activating

proteases, and (4) determine whether proteases known to activate PAR₂ induce intestinal inflammation.

Materials and Methods

PAR₂ Agonists and Protease Inhibitors

Pancreatic trypsin I/II was from Sigma Chemical Co (T1426, 10,000 U/mg; St Louis, MO), and recombinant human mast cell tryptase was from Promega (34.5 U/mg; Madison, WI). Trypsinogen IV and prop23 were expressed in *Escherichia coli*, purified by affinity chromatography, and activated by enterokinase.²¹ Purified trypsin IV (145 U/mg) and p23 (165 U/mg) were used for all experiments. Trypsin IV and p23 activity was determined using the substrate tosyl-GPR-pNA (150 μmol/L, 100 mmol/L Tris-HCl, 1 mmol/L CaCl₂, pH 8, 25° C, with 1 U defined as the enzymatic activity converting 1 μmol of substrate per minute). PAR₂-activating peptide (AP; SLIGRL-NH₂, corresponding to the tethered ligand that is exposed by proteolytic activation) and the inactive reverse peptide (LRGILS-NH₂, control) were from CPC Scientific (San Jose, CA). SBTI was from Fluka (93619) and Sigma Chemical Co (T9128) (Sigma, St. Louis, MO). FUT-175 was from Calbiochem (San Diego, CA). MPI-0442352 was a gift from C. Pleiman (Myriad Genetics, Salt Lake City, UT). A β strand template library^{22–24} was used to develop the tryptase inhibitor MPI-0442352 (mol wt, 784.94 with C₄₀H₄₈N₈O₇S). Tryptase inhibition was quantified using the release of pNA from the chromogenic substrate S-2366, L-pyroGlu-Pro-Arg-pNA (diaPharma, West Chester, OH), monitored at 405 nm and has been described.²⁵ In brief, MPI-0442352 (0–600 nmol/L) and human lung tryptase (0.2 nmol/L, in Tris, pH 8.0) (Elastin Products, Owensville, MO) were incubated at room temperature for 30 minutes, and S-2366 (50–800 μmol/L) was added to initiate the reaction. MPI 0443252, purified to >95%, had a K_i of 90 nmol/L.

Animals

The Institutional Animal Care and Use Committee of the University of California at San Francisco approved all animal protocols. Mice in which the *Par*₂ gene, also known as *F2r11*, was deleted²⁶ were maintained in a 50/50 mixed 129SVJae/C57BL6 strain background. Littermate *Par*₂^{+/+}, *Par*₂^{+/-}, and *Par*₂^{-/-} mice from heterozygote crosses were used for studies. *DPPI*^{+/+} and *DPPI*^{-/-} mice from Dr Timothy Ley²⁷ were backcrossed 10 generations into a C57BL/6 background and bred as homozygous knockouts. C57/Bl6 mice were from Jackson Laboratory (Davis, CA). Mice were maintained under climate-controlled (22 ± 4°C) and light-controlled (12-hour light/12-hour dark cycle) conditions in a barrier facility. Male and female mice were studied at 6–12 weeks of age.

Induction of Intestinal Inflammation With TxA

C difficile TxA was purified as described.²⁸ Genetically altered mice were caged before experimentation and studied in a blinded manner. Mice were fasted overnight but allowed access to water. They were anesthetized with isoflurane, and TxA (0.5 or 1 μg, whichever was a submaximal dose in the strain of mice selected for study) or vehicle (0.05 mol/L Tris, pH 7.4, control) in 100 μL was injected into ileal loops as described.²⁹ The animals received buprenorphine (0.05 mg/kg subcutaneously), were allowed to regain consciousness, and were killed after 3 hours (pentobarbital sodium 200 mg/kg intraperitoneally). Intestinal loops were removed, and the loop length and weight were recorded. Data are reported as milligrams per centimeters and as a percentage of the wild-type or positive control group. Loop fluid and a portion of the ileum were frozen separately for measurement of MPO activity. The remaining tissue was fixed in 10% formalin and paraffin embedded, after which sections were stained with H&E for histologic grading. Some C57/Bl6 mice received FUT-175 (10 mg/kg, in 0.25 mmol/L Tris, pH 7.4, injected into the loop 15 minutes before addition of TxA or vehicle [control]), MPI-0442352 (15 mg/kg, in dimethylacetamide/PEG300/EtOH/water at 3%/40%/

12%/45% intraperitoneally 30 minutes before TxA or vehicle), SBTI (10 or 20 mg in 0.025 mol/L Tris, pH 7.4, injected into the loop 15 minutes before TxA or vehicle), or corresponding vehicle. The number of animals used was 4–13 in control groups and 6–26 in experimental groups.

Induction of Intestinal Inflammation With Activators of PAR₂

Ileal loops were surgically prepared in anesthetized wild-type mice as described. AP (6.6 μ g/mouse), trypsin II (12 μ g/mouse), trypsin (4 μ g/mouse), trypsin IV (25 μ g/mouse), p23 (2.3 μ g/mouse), reverse peptide, or vehicle (0.05 mol/L Tris, pH 7.4, controls) (all in 100 μ L) was injected into the loop. Inflammation was assessed after 4 hours. In a separate group of experiments, mice were pretreated with the neurokinin 1 receptor antagonist SR140333 (Dr Xavier Edmonds-Alt, Sanofi Recherche, Montpellier, France) (2 mg/kg) or its carrier (1% dimethyl sulfoxide) intraperitoneally (0.2 mL) 60–90 minutes before formation of ileal loops and injection with AP. The number of mice used was 6 in each group.

MPO Assay

Both tissue and loop fluid were assayed. Samples were homogenized and sonicated, and the homogenate was centrifuged (12,000g, 15 minutes). MPO activity in the supernatant was quantified in triplicate at 2 dilutions with a microtiter plate assay using 5-*O*-dianiside (Aldrich, Milwaukee, WI) as the substrate and human neutrophil MPO (Calbiochem, San Diego, CA) as a standard.²⁹ Supernatants were assayed for protein in triplicate with a bicinchoninic acid microtiter plate assay (Pierce, Rockford, IL). Results obtained with tissue are expressed as units of MPO per milligram of protein. Results obtained with fluid are expressed as units of MPO per milliliter of fluid. Results between groups are compared as percentages.

Microscopy and Histologic Scoring

Sections were observed by using a Zeiss Axioplan microscope (Zeiss, Thornwood, NJ) and a Zeiss Fluor \times 20 (numerical aperture, 1.0) or Plan Aplanachromat \times 40 (numerical aperture, 1.4). Images were imported via a SPOT Insight camera (Diagnostic Instruments, Sterling Heights, MI). Contrast and brightness were adjusted using Adobe Photoshop CS (Adobe Systems, Mountain View, CA). The severity of inflammation was scored in coded slides by a histologist blinded to experimental groups on a scale of 1 (mild) to 3 (severe) for epithelial damage, edema, and neutrophil infiltration as described.²⁸

Localization of PAR₂

In a separate series of experiments, TxA (1.0 μ g) or vehicle (control) was injected into ileal loops formed in C57/Bl6 wild-type mice as described. After 4 hours, the loop tissue was fixed in 4% paraformaldehyde in 100 mmol/L phosphate-buffered saline, pH 7.4, overnight at 4°C, washed, placed in 30% sucrose for 16 hours at 4°C, and embedded in OCT. Frozen sections (10 μ m) were incubated in phosphate-buffered saline containing 0.1% Triton X-100 and 5% normal goat serum. Sections were incubated with rabbit anti-mouse PAR₂ antiserum (9717,30 generated by CURE, University of California at Los Angeles; 1:400, 16 hours at 4°C) and washed. For immunofluorescence, sections were incubated with a goat anti-rabbit anti-immunoglobulin G conjugated to fluorescein isothiocyanate (FITC; 1:200, 2 hours; Jackson ImmunoResearch, West Grove, PA).³¹ Tissue sections in which the primary antibody was omitted served as controls. Single confocal sections were acquired using a Zeiss LSM510 Meta equipped with an argon laser and an Axiovert 200M microscope using a Plan Aplanachromat \times 20 (numerical aperture, 0.80), 488-nm excitation, and a long-pass 505 filter. Acquisition parameters were adjusted to be less sensitive in the PAR₂ antiserum-stained tissue to ensure that the brighter-staining TxA-treated tissue was not at saturation and more sensitive in the secondary antibody only tissue to enable visualization of the tissue. Images used were unmodified. Pixel

intensity was quantified using LSM510 software on unmodified images acquired using identical parameters.

Cell Culture and Treatment of NCM460 Cells With TxA

NCM460 cells, an epithelial cell line from normal human colon,⁶ were maintained in Ham's F-12 medium with 20% heat-inactivated fetal bovine serum, 0.5 mg/mL insulin-transferrin-selenium (Invitrogen, Carlsbad, CA), 0.4 mg/mL hydrocortisone (Sigma Chemical Co), and 0.58 $\mu\text{g}/\text{mL}$ L-glutamine in 95% air/5% CO_2 at 37°C. Cells were plated at $5 \times 10^5/\text{mL}$ onto plastic for polymerase chain reaction (PCR) and onto glass coverslips for measurement of intracellular calcium concentration ($[\text{Ca}^{2+}]_i$). After 48 hours, they were washed with phosphate-buffered saline containing Ca^{2+} and Mg^{2+} and placed in Ham's F-12 containing 0.1% bovine serum albumin. Cells were treated at 37°C with vehicle or TxA (5 nmol/L) for 0, 6, or 24 hours or with vehicle or TxA (5 $\mu\text{mol}/\text{L}$) for 30–360 minutes. Vehicle consisted of the equivalent dilution of Tris 0.05 mol/L in NCM culture medium (1/1300 or 1/1,300,000 for 5 $\mu\text{mol}/\text{L}$ and 5 nmol/L of TxA, respectively). Cells were then used for PCR and Ca^{2+} signaling assays.

Measurement of $[\text{Ca}^{2+}]_i$

NCM460 cells were washed and incubated in Hanks' balanced salt solution with 0.1% bovine serum albumin, 20 mmol/L HEPES (pH 7.4), and 5 $\mu\text{mol}/\text{L}$ of fura-2/AM (Molecular Probes, Eugene, OR) for 45–60 minutes at 37°C. Washed coverslips were mounted in an open chamber at 37°C. Fluorescence of individual cells was measured at 340 nm and 380 nm excitation and 510 nm emission using a Zeiss Axiovert microscope, an ICCD video camera (Stanford Photonics, Stanford, CA), and a video microscopy acquisition program (Axon Instruments, Inc, Union City, CA). Test substances (50 μL injection) were added to the chamber. Results are expressed as the 340/380-nm emission ratio, which is proportional to the $[\text{Ca}^{2+}]_i$. The magnitude of the response to PAR_2 -AP was determined by subtraction of the baseline response; thus, each cell served as an internal control.

Real-Time Quantitative PCR Analysis

Total RNA was extracted from NCM460 cells using the RNeasy kit (Qiagen, Valencia, CA), treated with deoxyribonuclease (Ambion, Austin, TX) for 60 minutes and repurified. The reverse-transcriptase reaction (100 μL final using 1 μg of RNA) was conducted as follows: 25°C for 10 minutes, 48°C for 30 minutes, and 99°C for 5 minutes using random hexamers. The PCR reaction mixture contained 25 μL of 2 \times Universal PCR master mix, 0.5 μL of template complementary DNA, and 2.5 μL of primers/probe (Applied Biosystems, Foster City, CA) in 50 μL in a 96-well hard-shell reaction plate (MJ Research, Waltham, MA). Reactions were amplified using a continuous fluorescence detector (DNA Engine Opticon 2; MJ Research) and performed in triplicate. For PAR_2 and β -glucuronidase, primers and probes were from Applied Biosystems (cat. no. Hs001173741 and Hs99999908, respectively). Primers and probes for mesotrypsin/trypsin IV were from Applied Biosystems (primer/probes sequences were as follows: forward, 5'-ccaccctaaatacaacaggacac-3'; reverse, 5'-tggacacgeggacatt-3'; probe, 5'atcatgctgatcaactctctcacctg-3'). The relative quantity messenger RNA was obtained of by the comparative threshold cycle (C_t) method with β -glucuronidase as an endogenous control. The C_t indicates the cycle number at which the amount of amplified target reaches a fixed threshold above background. Opticon Monitor Analysis software (version 2.02; MJ Research) was used to calculate a C_t value for each reaction, which was normalized for amplification by subtracting the C_t value of β -glucuronidase. The fold increase was calculated relative to the C_t value of unstimulated cells.

Statistics

Results are reported as means \pm SE. Data were compared using analysis of variance and multiple comparison testing with the Student–Newman–Keuls *t* test or Student *t* test, with *P* < .05 considered significant.

Results

Deletion of PAR₂ Ameliorates TxA-Induced Fluid Secretion and Granulocyte Infiltration

We compared enteritis with a half-maximal dose of TxA (0.5 μ g for the *Par*₂^{+/+} mice) in *Par*₂^{+/+}, *Par*₂^{+/-}, and *Par*₂^{-/-} mice using accumulation of fluid in the intestinal lumen and MPO activity in both the intestinal wall and fluid as end points. Basal fluid secretion in vehicle-treated control loops was similar in *Par*₂^{+/+}, *Par*₂^{+/-}, and *Par*₂^{-/-} mice (55 \pm 4, 48 \pm 4, and 53 \pm 9 mg/cm, respectively) (Figure 1A). In *Par*₂^{+/+} mice, TxA significantly increased secretion from basal levels by 2.5-fold (135 \pm 8 mg/cm; *P* < .05). In comparison, TxA-induced secretion in *Par*₂^{+/-} mice was decreased by ~15% (116 \pm 10 mg/cm), and TxA-induced secretion in *Par*₂^{-/-} mice was significantly decreased by ~20% (109 \pm 8 mg/cm; *P* < .05) (Figure 1A). Granulocyte infiltration (assessed by MPO assays of tissue and loop fluid) was similar in controls among *Par*₂^{+/+}, *Par*₂^{+/-}, and *Par*₂^{-/-} mice (tissue: 0.07 \pm 0.04, 0.07 \pm 0.03, and 0.05 \pm 0.03 U MPO/mg protein; loop fluid: 0.08 \pm 0.02, 0.07 \pm 0.03, and 0.07 \pm 0.04 U MPO/mL, respectively) (Figure 1B and C). TxA also caused a significant 30-fold increase in tissue MPO in *Par*₂^{+/+} mice (tissue: 2.15 \pm 0.22 U MPO/mg protein; loop fluid: 0.8 \pm 0.02 U MPO/mL; *P* < .05). Compared with wild-type mice, *Par*₂^{+/-} and *Par*₂^{-/-} mice had decreased responses to TxA, with a 15% and 49% reduction in tissue MPO and a 20% and 56% decrease in fluid MPO, respectively (tissue: 1.85 \pm 0.22 and 1.10 \pm 0.14 U MPO/mg protein; loop fluid: 0.64 \pm 0.1 and 0.35 \pm 0.06 U MPO/mL, respectively) (Figure 1B and C). Once again, these decreases were statistically significant in the *Par*₂^{-/-} mice (*P* < .05). Thus, PAR₂ deletion decreases TxA-induced intestinal secretion and granulocyte infiltration in a gene/dose-dependent manner, with heterozygote mice having a smaller decrease from wild-type mice than knockout mice. Deletion of PAR₂ did not eliminate inflammation, which in this case must be due to activation of other receptors.

Deletion of PAR₂ Ameliorates TxA-Induced Pathologic Changes

Histologic assessment confirmed that epithelial damage, edema, and neutrophil infiltration induced by TxA were diminished in *Par*₂^{+/-} mice and significantly decreased in *Par*₂^{-/-} mice relative to *Par*₂^{+/+} animals (Table 1 and Figure 2; *P* < .05). In *Par*₂^{-/-} mice treated with TxA, there was significantly diminished epithelial damage, edema, and infiltration of neutrophils (*P* < .05) and a striking retention of mucus in goblet cells. Thus, deletion of PAR₂ diminishes all characteristics of TxA-induced enteritis examined.

Deletion of DPPI Ameliorates TxA-Induced Fluid Secretion and Granulocyte Infiltration

Responses of *DPPI*^{+/+} and *DPPI*^{-/-} mice to a half-maximal dose of TxA (1 μ g in this strain, *DPPI*^{+/+} mice were less sensitive to TxA) showed that basal fluid secretion did not differ between *DPPI*^{+/+} (46 \pm 2 mg/cm) and *DPPI*^{-/-} mice (54 \pm 4 mg/cm) (Figure 3A). TxA caused a robust increase in intestinal secretion over that seen with vehicle control (139 \pm 7 mg/cm), similar to that seen in *Par*₂^{+/+} mice (Figure 1A). In *DPPI*^{-/-} animals, intestinal secretion induced by TxA was significantly reduced to 78% of that seen in wild-type animals (109 \pm 6 mg/cm; *P* < .05). Basal granulocyte infiltration, as determined by MPO assays, was similar in *DPPI*^{+/+} and *DPPI*^{-/-} animals (tissue: 0.06 \pm 0.01 and 0.05 \pm 0.01 U MPO/mg protein; loop fluid: 0.03 \pm 0.01 and 0.03 \pm 0.01 mU MPO/mL, respectively). Granulocyte infiltration in response to TxA, as determined by tissue MPO, was not different between the strains (1.9 \pm 0.2 and 1.8 \pm 0.2 mU MPO/mg protein in *DPPI*^{+/+} and *DPPI*^{-/-} animals, respectively) (Figure

3B), but fluid MPO was significantly decreased; levels seen in *DPPI*^{-/-} mice were 45% of those in *DPPI*^{+/+} mice (1.7 ± 0.2 and 0.79 ± 0.1 mU MPO/mL in *DPPI*^{+/+} and *DPPI*^{-/-} animals, respectively; $P < .05$) (Figure 3C). Thus, deletion of DPPI decreases TxA-induced intestinal secretion and fluid MPO but not tissue MPO. Again, residual inflammation induced by TxA must be due to other receptors and agonists that participate in host responses.

Deletion of DPPI Ameliorates TxA-Induced Pathologic Changes

Histologic assessment showed that *DPPI*^{+/+} and *DPPI*^{-/-} mice appeared similar under basal conditions (Figure 4 and Table 2). In *DPPI*^{+/+} mice, TxA caused pathologic changes, including a loss of villi, edema, neutrophil infiltration, and necrosis, that were diminished in *DPPI*^{-/-} mice, with a significantly reduced edema ($P < .05$) and a reduced total score. Thus, deletion of DPPI diminishes the characteristics of TxA-induced enteritis.

Tryptase and Trypsin Inhibitors Ameliorate TxA-Induced Intestinal Secretion and Granulocyte Infiltration

We determined if pharmacologic blockade of tryptase or trypsin would diminish TxA responses in wild-type C57/B16 mice. In control experiments, mice treated with vehicle before a submaximal dose of TxA ($1.0 \mu\text{g}$ in C57/B16) had a strong secretory response (122 ± 7 mg/cm) (Figure 5A). Pretreatment with the tryptase inhibitors FUT-175 or MPI-0442352 significantly decreased secretion by 24% and 28%, respectively (93 ± 9 and 89 ± 13 mg/cm, respectively; $P < .05$). Pretreatment with 10 or 20 mg SBTI also significantly decreased secretory responses to TxA by 62% and 38%, respectively, with a return to basal or almost basal levels of secretion (47 ± 3 and 76 ± 5 mg/cm, respectively; $P < .05$). Tissue MPO activity was prominent in mice treated with vehicle before TxA (1.72 ± 0.1 U MPO/g protein) (Figure 5B) but was significantly decreased by 47% and 31%, respectively, in groups pretreated with FUT-175 or MPI-0442352 (0.92 ± 0.1 and 1.19 ± 0.12 U MPO/mg protein, respectively; $P < .05$). Pretreatment with 10 and 20 mg SBTI also significantly decreased tissue MPO by 56% and 30%, respectively (0.76 ± 0.16 and 1.21 ± 0.2 U MPO/mg protein, respectively; $P < .05$), but increased basal tissue MPO. Fluid MPO activity was increased in mice treated with vehicle before TxA (0.45 ± 0.09 U MPO/mL with TxA) (Figure 5C). Pretreatment with FUT-175 and MPI-0442352 significantly diminished responses by 71% and 38%, respectively (0.13 ± 0.04 and 0.28 ± 0.06 U MPO/mL, respectively; $P < .05$). We were unable to quantify fluid MPO in SBTI-pretreated mice, because SBTI interacted with the substrate to cause spurious results in the MPO assay. Thus, inhibitors of tryptase and trypsin suppress TxA-induced ileitis, but again some residual inflammation remained in many experimental groups.

Tryptase and Trypsin Inhibitors Ameliorate TxA-Induced Pathologic Changes

In vehicle-treated control mice, TxA caused marked epithelial damage, edema, and neutrophil infiltration (Figure 6 and Table 3). Pretreatment with FUT-175 significantly decreased both epithelial damage and total histologic score ($P < .05$), but MPI-0442352 did not alter the pathologic changes induced by TxA. SBTI significantly decreased epithelial damage, edema, and total score. Granulocyte infiltration occurred in limited areas (Figure 6), which was not reflected in the total score (Table 3).

TxA Up-Regulates Immunoreactive PAR₂ in the Mouse Ileum

We determined the effect of TxA on levels of immunoreactive PAR₂ in the intestine. Under basal conditions, PAR₂ was detected on colonocytes in the ileum (Figure 7A). In mice treated with $1 \mu\text{g}$ TxA for 4 hours, the intensity of PAR₂ immunoreactivity in enterocytes was significantly increased (Figure 7B). PAR₂ staining in tissue obtained from mice treated with buffer had $100,785 \pm 48,537$ pixels above baseline, whereas PAR₂ staining in tissue obtained from mice treated with TxA had $346,401 \pm 58,338$ pixels above baseline ($P < .05$). Tissue

sections incubated only with anti-rabbit immunoglobulin G conjugated to FITC had no staining (Figure 7C and D). To enable visualization of the tissue, we acquired these images with a higher gain than that used for sections stained for PAR₂ (compare black levels in Figure 7A and B vs Figure 7C and D). Thus, exposure to TxA significantly increased PAR₂ expression.

TxA Enhances PAR₂ and Trypsin IV Expression and Activity of PAR₂ in Human Colonocytes

To evaluate in more detail the effects of TxA on expression and activity of PAR₂ and PAR₂-activating proteases, we exposed NCM460 cells to TxA or vehicle (control). We assessed expression of PAR₂ and trypsin IV messenger RNA using real-time quantitative PCR with a C_t method and β -glucuronidase as a control. Trypsin IV is an extrapancreatic trypsin that is expressed in epithelial cell lines and normal human intestine and can activate PAR₂.⁶ Results above threshold were obtained after 28–30 cycles. Exposure to TxA (5 μ mol/L) caused a statistically significant increase in expression of PAR₂ messenger RNA to 174% of control levels at 2 hours and 199% at 4 hours (Figure 8A) but after 6 hours declined to 136% of control ($P < .05$). TxA also caused a striking increase in trypsin IV expression to 149% after 2 hours and 211% after 6 hours (Figure 8B). Thus, TxA causes a doubling in RNA levels of PAR₂ and trypsin IV, which remain elevated for a prolonged period.

To assess whether TxA up-regulated functional PAR₂, we analyzed PAR₂-dependent signaling in NCM460 cells by measuring changes in [Ca²⁺]_i in response to AP, a PAR₂-selective agonist. Because TxA may cause transient changes in [Ca²⁺]_i, we determined that levels had returned to basal before stimulation with AP. Prolonged exposure to TxA did not significantly increase the baseline [Ca²⁺]_i (0.76 \pm 0.4, 0.57 \pm 0.1, 0.69 \pm 0.3, and 0.81 \pm 0.4 in cells exposed to vehicle, 5 nmol/L TxA for 6 hours, 5 nmol/L TxA for 24 hours, and 5 μ mol/L TxA for 1 hour with 24-hour recovery, respectively). In comparison with vehicle (peak baseline, 0.087 \pm 0.006), exposure to TxA (5 nmol/L) for 6 hours significantly increased responses to AP (10⁻⁵ mol/L) by 190% (0.166 \pm 0.018; Figure 8C; $P < .05$). This increase was stable and did not rise further after 24 hours with TxA, when it was 184% of control (0.160 \pm 0.013). To determine if a prolonged effect would occur, we incubated cells with a higher dose of TxA (5 μ mol/L) for 1 hour and tested PAR₂ Ca²⁺ responses after a 24-hour recovery. Responses were 156% of control (0.136 \pm 0.013; $P < .05$). Thus, exposure of colonocytes to TxA causes a marked increase in PAR₂ signaling that remains for 24 hours after TxA is withdrawn.

Activation of PAR₂ Induces Inflammation in the Intestine

Because TxA-induced inflammation was diminished in mice with deletion or inhibition of PAR₂ or enzymes that activate PAR₂ and because TxA increased PAR₂ expression and function in cell lines and animals, we determined whether PAR₂ activation alone would induce ileitis. Mice were treated with AP, trypsin, pancreatic trypsin II, extrapancreatic human trypsin IV, and p23, a rat trypsin isozyme that resembles trypsin IV in its resistance to endogenous inhibitors.²¹ Intestinal secretion and MPO activity were measured after 4 hours. AP significantly stimulated intestinal secretion (111 \pm 20 mg/cm; Figure 9A; $P < .05$ vs vehicle control). All enzymes tested, trypsin, trypsin II, trypsin IV, and p23, significantly increased intestinal secretion, albeit to a lower level than AP (76 \pm 4, 78 \pm 4, 80 \pm 11, and 89 \pm 7 mg/cm, respectively; Figure 9A). AP also significantly stimulated granulocyte infiltration (tissue MPO, 0.20 \pm 0.03 U MPO/mg protein; fluid MPO, 0.26 \pm 0.02 U MPO/mL fluid; $P < .05$ vs vehicle control). All enzymes tested, trypsin, trypsin II, trypsin IV, and p23, significantly increased tissue MPO levels (0.18 \pm 0.05, 0.23 \pm 0.04, 0.17 \pm 0.01, and 0.19 \pm 0.03 U MPO/mg protein, respectively; Figure 9B; $P < .05$). Fluid MPO levels were maximally increased with trypsin and trypsin II (0.22 \pm 0.04 and 0.26 \pm 0.07 U MPO/mL fluid, respectively; Figure 9C; $P < .05$ vs vehicle control), while trypsin IV and p23 induced a weaker response (0.14 \pm 0.29 and 0.15 \pm 0.04 U MPO/mL fluid, respectively; Figure 9C; $P < .05$ vs vehicle control). Reverse peptide did not induce a significant inflammatory response (results not shown; fluid

secretion, 46.4 ± 6.6 mg/cm; tissue MPO, 0.07 ± 0.02 U/mg protein; fluid MPO, 0.03 ± 0.01 U/mL). Thus, AP and the enzymes that activate PAR₂ can induce ileitis.

PAR₂-Induced Ileitis Occurs via Neurogenic Mechanisms

Because substance P and the neurokinin 1 receptor in large part mediate TxA-induced ileitis, ^{28,32} we also examined their role in PAR₂-induced inflammation. We treated mice with the neurokinin 1 receptor antagonist SR140333 or vehicle (control) before induction of ileitis with AP. Mice treated with vehicle had robust levels of intestinal secretion and tissue and fluid MPO activity (intestinal secretion, 74 ± 0.03 mg/cm; tissue MPO, 0.32 ± 0.05 U/mg protein; fluid MPO, 0.37 ± 0.09 U/mL fluid; Figure 10A–C). SR140333 decreased intestinal secretion to basal levels and significantly diminished tissue and fluid MPO activity by 59% and 71%, respectively (intestinal secretion, 47 ± 0.02 mg/cm; tissue MPO, 0.13 ± 0.01 U/mg protein; fluid MPO, 0.11 ± 0.04 U/mL fluid; $P < .05$; Figure 10A–C; $P < .05$). Thus, PAR₂-induced ileitis occurs via neurogenic mechanisms involving activation of the neurokinin 1 receptor.

Discussion

In our experiments, deletion of PAR₂ resulted in diminished inflammatory responses to TxA, as reflected by decreases in intestinal secretion, granulocyte infiltration into tissues and luminal fluid, and improved histologic assessment. Deletion and inhibition of PAR₂-activating proteases also reduced TxA-induced ileitis. DPPI-deficient mice, which have reduced levels of active trypsin, had decreased intestinal secretion, fluid MPO activity, epithelial damage, and edema. The serine protease inhibitors FUT-175 and MPI-0442352, as well as SBTI, all diminished inflammation in response to TxA. TxA increased immunoreactive PAR₂ on enterocytes of the ileum, while exposure of human colonocytes to TxA sensitized responses to PAR₂ agonists and up-regulated messenger RNA for PAR₂ and trypsin IV. PAR₂ activators, such as AP, trypsin, and trypsins, also induced ileitis by a mechanism that depends on activation of the neurokinin 1 receptor. Together, these results indicate that PAR₂ and enzymes that can activate PAR₂ play a proinflammatory role in enteritis induced by TxA from *C. difficile*. The mechanism may involve both activation of existing PAR₂ and up-regulation of PAR₂ and trypsin IV expression in enterocytes. Moreover, PAR₂-induced inflammation occurs via a neurogenic mechanism involving activation of the neurokinin 1 receptor.

Contribution of PAR₂ to *C. difficile* TxA-Induced Enteritis

Although the inflammatory response to TxA was strong in *Par*₂^{+/+} mice, it diminished significantly in *Par*₂^{-/-} mice and was intermediate in *Par*₂^{+/-} mice, suggesting a gene/dosage effect. These results suggest that PAR₂ plays a proinflammatory role in *C. difficile* enteritis. In support of this role for PAR₂, deletion of PAR₂ also protects against colitis induced by *Citrobacter rodentium*, allergic inflammation of the airways, and arthritis.^{33–36} In contrast, pretreatment with PAR₂-AP protects against chemically induced colitis due to the release of calcitonin gene-related peptide.¹¹ Although deletion of PAR₂ significantly diminished MPO, TxA-induced pathologic changes were more completely blocked. This result may be due to the difference in recruitment versus activation of neutrophils. Activation of neutrophils is responsible for TxA-induced tissue damage, and an MPO assay would not discriminate between recruitment and activation of neutrophils.

We found that PAR₂ activation by AP, trypsin, and several isozymes of trypsin (bovine pancreatic trypsin I/II, human trypsin IV, and rat p23) induced ileitis, with statistically significant increases in intestinal secretion and tissue and fluid MPO activity. Neurogenic mechanisms participate in PAR₂-induced inflammation, because our results show that blockade of neurokinin 1 receptors prevents AP-induced ileitis. Thus, the participation of PAR₂ in inflammatory responses to TxA is likely due, in part, to release of substance P and

activation of the neurokinin 1 receptor. These results confirm observations in the intestine and skin that PAR₂ agonists cause inflammation by a neurogenic mechanism.^{12,37} Similar neurogenic mechanisms also mediate TxA-induced enteritis, because antagonism or deletion of substance P and calcitonin gene-related peptide prevents TxA-induced enteritis.^{28,29,32,38,39} During TxA-induced enteritis, substance P and calcitonin gene-related peptide are up-regulated both locally and in dorsal root ganglia, emphasizing their role in the inflammatory response.^{39,40} Neurokinin 1 receptors are also up-regulated and activated during TxA-induced enteritis.³⁸ PAR₂ activation of enteric neurons increases short-circuit current, indicative of chloride ion secretion,¹⁷ which would lead to intestinal secretion, which we found diminished with deletion of PAR₂. Thus, both extrinsic and intrinsic neurons can mediate modulation of the inflammatory response to TxA by PAR₂.

Further support for involvement of PAR₂ in *C difficile* enteritis is provided by our finding that TxA up-regulates PAR₂ immunoreactivity in enterocytes in the ileum, PAR₂ responsiveness, and PAR₂ and trypsin IV messenger RNA in colonocytes. These findings also suggest that the enterocyte is one of the key cells that participate in modulation of TxA responses by PAR₂. Our results are consistent with other reports that PAR₂ is up-regulated in cell lines by specific inflammatory mediators as well as in inflamed tissues.^{33,41–43} PAR₂ up-regulation during *C difficile* infection could magnify the proinflammatory actions of proteases on epithelial cells, because activation of PAR₂ on enterocytes disrupts tight junctions to increase paracellular permeability, which can result in bacterial translocation and colonic inflammation.^{1,7} TxA similarly disrupts tight junctions of enterocytes.¹⁵

PAR₂ has other roles in inflammation that may participate in TxA-induced enteritis, but these have not been examined. For example, PAR₂ is present on endothelial cells and neutrophils, and PAR₂ agonists promote interleukin-6 secretion, L-selectin shedding by granulocytes, and leukocyte rolling and adhesion.^{26,44,45} Recruited neutrophils secrete proteases, including neutrophil elastase, that activate PAR₂ on epithelial and nonepithelial cells to induce release of interleukin-8 and other cytokines important in TxA-induced inflammation.⁴⁶ These potential mechanisms require further investigation.

Contributions of Proteases to *C difficile* TxA-Induced Enteritis

Our use of knockout mice and protease inhibitors to study the involvement of proteases in TxA-induced colitis showed that although deletion of DPPI reduced TxA-induced intestinal secretion, fluid MPO activity, and tissue edema, tissue MPO activity, neutrophil infiltration, and epithelial damage were unaltered. Thus, DPPI may influence the secretion of fluid and neutrophils into the lumen, without a marked effect on migration and activation of granulocytes in the tissues. Therefore, deletion of DPPI protects against TxA-induced enteritis, but to a smaller extent than PAR₂ deletion. This is not surprising, because PAR₂ is activated by several proteases,^{2,3,5} and *DPPI*^{-/-} mice retain ~25% of normal tryptase levels.⁸

Understanding the mechanism by which deletion of DPPI protects against TxA-induced enteritis is complicated by the fact that deletion of DPPI affects the activity of several proteases, only some of which can activate PAR₂. DPPI knockout mice show diminished activity of tryptase, neutrophil elastase, and proteinase 3 but are completely deficient in activity of cathepsin G, mast cell chymase, and the granzymes A and B of cytotoxic lymphocytes.^{8,27,47}

The serine protease inhibitors FUT-175 and MPI-0442352 inhibited TxA-induced enteritis, as evidenced by decreased intestinal fluid secretion and granulocyte infiltration into tissues and the intestinal lumen. FUT-175, originally described as a synthetic inhibitor of complement and serine proteases such as thrombin and trypsin, is a potent and selective inhibitor of human tryptase, with a K_i of 95 pmol/L.²⁰ At 10 mg/kg, the dose used in this study, FUT-175 inhibits

scratching induced by tryptase but not by histamine or serotonin in mice.⁴⁸ Our results obtained with FUT-175 may be largely due to tryptase inhibition, because results obtained with an alternative human tryptase inhibitor, MPI-0442352, were similar, although less striking. The broad-spectrum inhibitor of both tryptic and nontryptic serine peptidases, SBTI, caused the most dramatic decrease in intestinal secretion, granulocyte infiltration, and histologic changes in response to TxA. In support of our results, SBTI also reduces infectious colitis in mice.³⁶ The source and isozyme of tryptic peptidase that mediates TxA-induced enteritis remains to be determined. However, one possibility is trypsin IV, which we found to be up-regulated by TxA. Trypsin IV is a prominent protease that is widely expressed by extrapancreatic epithelial cells and tissues, including normal human colon, and can activate PAR₂.⁶ Because trypsin IV is resistant to endogenous polypeptide trypsin inhibitors and degrades these inhibitors, if released, it could signal for prolonged periods by cleaving PARs. Because there are marked differences in the human and mouse trypsin genes, we did not attempt to characterize the forms of trypsin that are up-regulated by TxA in mice. However, we did observe that both trypsin IV and p23, a rat trypsin isozyme that is also resistant to endogenous inhibitors,²¹ induced ileitis in mice.

Although deletion of PAR₂ and DPPI diminished responses to TxA, a substantial inflammatory response remained, which must be due to other mechanisms. These include other receptors known to participate in TxA-induced enteritis, such as the neurotensin receptor 1 and corticotrophin-releasing hormone receptors 1 and 2, both of which are up-regulated in response to TxA, and the CC chemokine receptor (CCR1).^{49–51}

In summary, our use of overlapping genetic and pharmacologic approaches showed that PAR₂ and its activators are proinflammatory in TxA-induced enteritis. Although each of these approaches by themselves has limitations, considered together our results show that deletion of PAR₂ and the proteases that activate this receptor protect against inflammatory responses to TxA. Intestinal inflammation mediated by PAR₂ involves a neurogenic mechanism. Moreover, TxA increases PAR₂ immunoreactivity, messenger RNA, and signaling in intestinal epithelial cells. Thus, our results show, for the first time, that PAR₂, DPPI, tryptase, and trypsin mediate the proinflammatory effects of TxA from *C difficile*. Because PAR₂ and tryptase are up-regulated in human intestinal disease, including irritable bowel syndrome and ulcerative colitis,^{52–54} PAR₂ and the enzymes that activate it may be a general mechanism in the pathophysiology of intestinal inflammation, including inflammatory bowel disease.

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Abbreviations used in this paper

AP, activating peptide; C_t, comparative threshold cycle; DPPI, dipeptidyl peptidase I; FITC, fluorescein isothiocyanate; MPO, myeloperoxidase; PAR₂, protease-activated receptor 2; PCR, polymerase chain reaction; SBTI, soybean trypsin inhibitor; TxA, toxin A.

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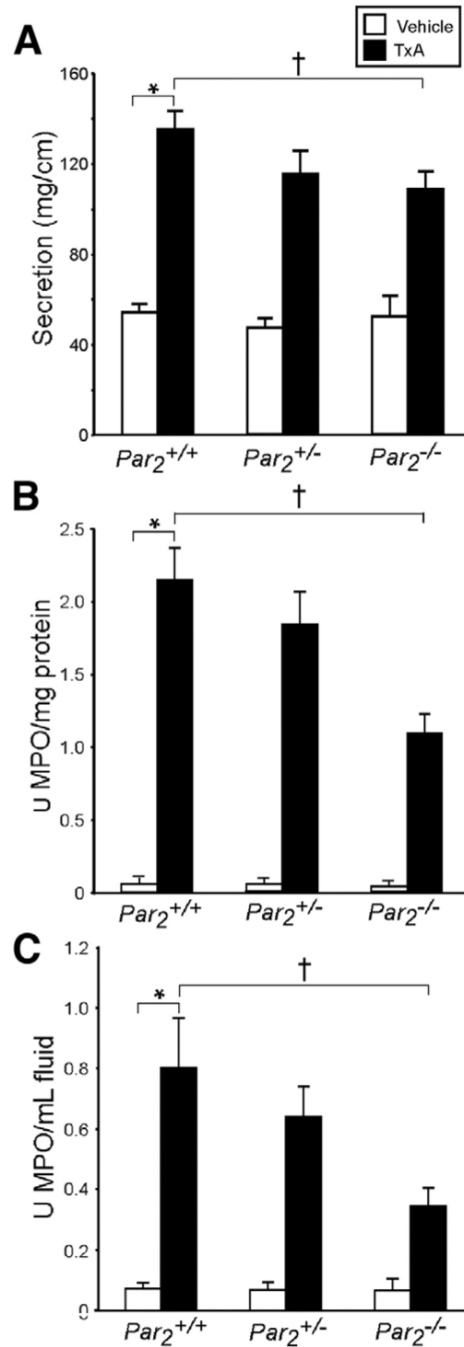


Figure 1.

Deletion of PAR₂ inhibits TxA-induced fluid secretion and granulocyte infiltration. Ileal loops were injected with vehicle (vehicle control, *open bars*) or 0.5 μ g TxA (*closed bars*). (A) Intestinal secretion expressed as milligrams per centimeter of loop. (B) Tissue MPO expressed as units MPO per milligram protein. (C) MPO in loop fluid expressed as units per milliliter fluid. * $P < .05$ between vehicle and TxA loops in Par₂^{+/+} mice. † $P < .05$ between results obtained with TxA in Par₂^{+/+} and Par₂^{+/-} or Par₂^{-/-} mice.

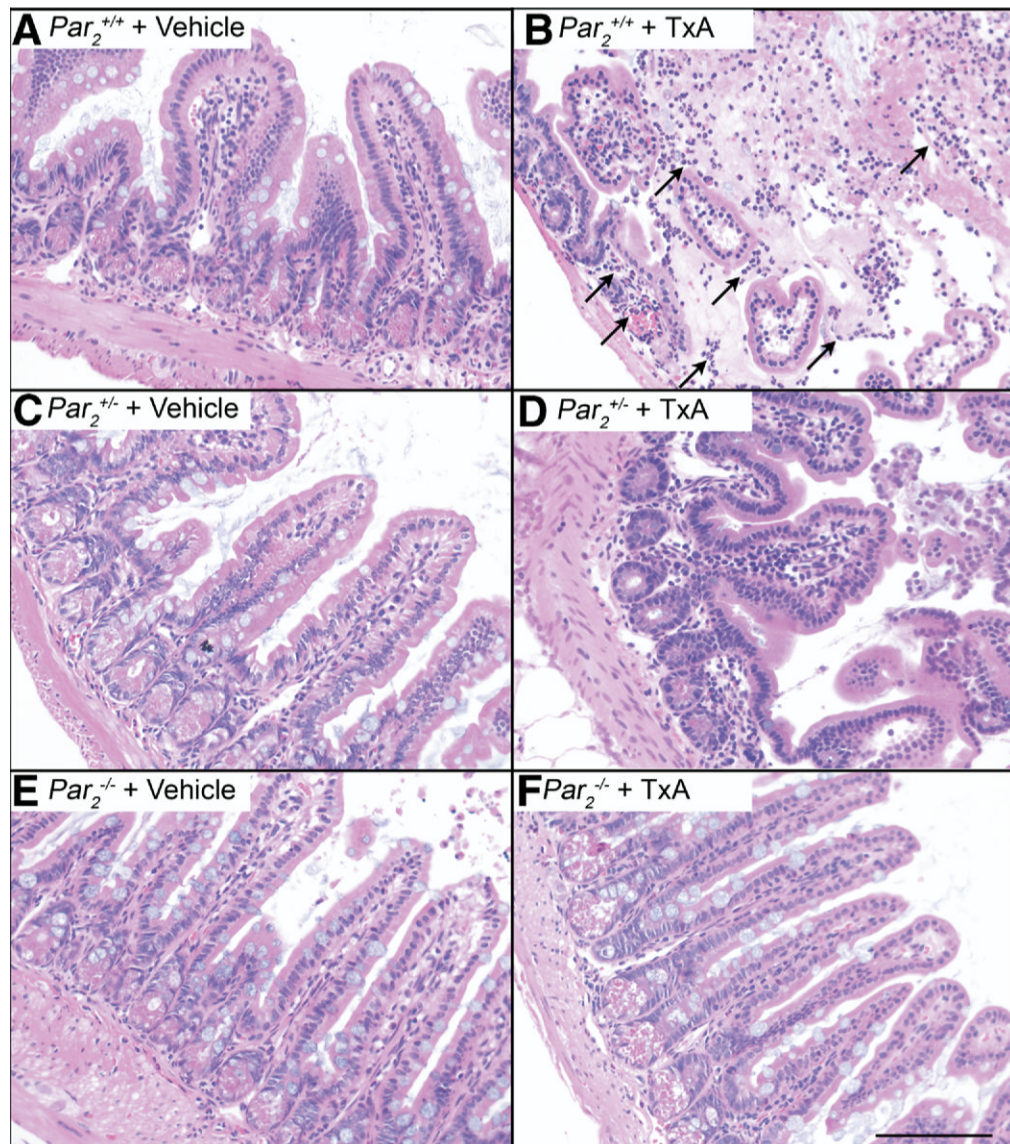


Figure 2. Deletion of PAR₂ inhibits TxA-induced enteritis. Images show the ileum. (A) $Par_2^{+/+}$ vehicle control, (B) $Par_2^{+/+}$ TxA, (C) $Par_2^{+/-}$ vehicle control, (D) $Par_2^{+/-}$ TxA, (E) $Par_2^{-/-}$ vehicle control, (F) $Par_2^{-/-}$ TxA. Note the normal villous structure in control groups. In $Par_2^{+/+}$ mice, TxA caused pathologic changes including loss of villi, infiltration of granulocytes, and severe necrosis (arrows). Scale bar = 100 μ m.

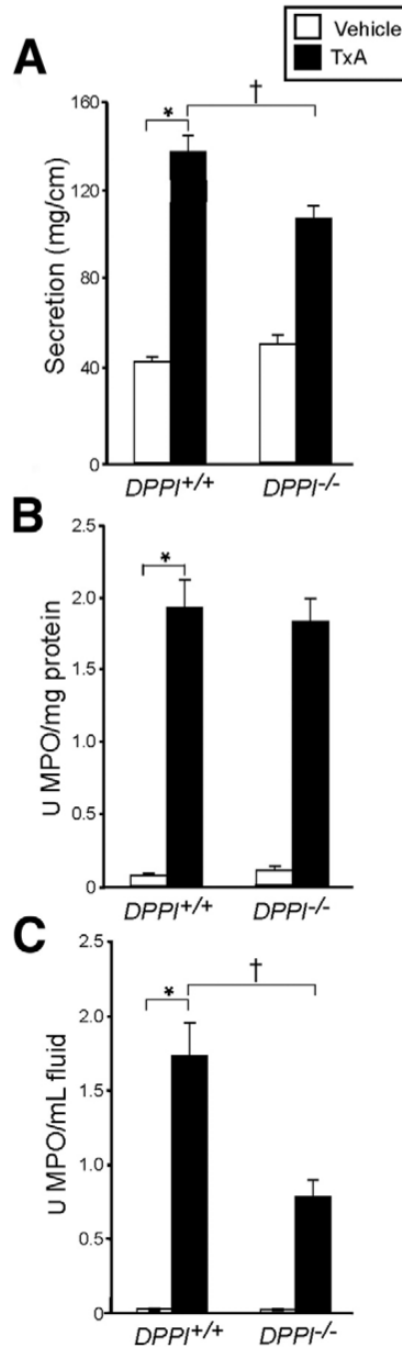


Figure 3.

Deletion of DPPI diminishes TxA-induced fluid secretion and MPO activity in loop fluid. Ileal loops were injected with vehicle (vehicle control, *open bars*) or 1.0 μg TxA (*closed bars*). (A) Intestinal secretion expressed as milligram per centimeter of loop. (B) Tissue MPO, expressed as units MPO per milligram protein. (C) MPO in loop fluid expressed as units per milliliter of fluid. * $P < .05$ between buffer and TxA loops in DPPI^{+/+} mice. † $P < .05$ between results obtained with TxA in DPPI^{+/+} and DPPI^{-/-} mice.

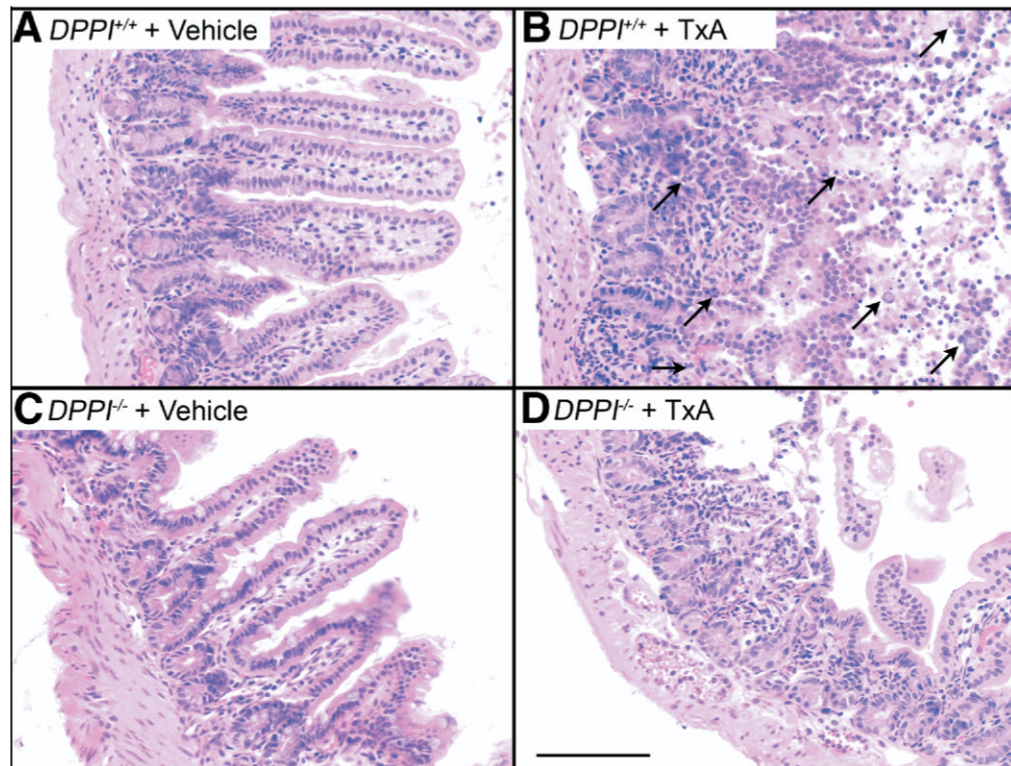


Figure 4. Deletion of DPPI inhibits TxA-induced enteritis. Images show the ileum. (A) *DPPI*^{+/+} vehicle control, (B) *DPPI*^{+/+} TxA, (C) *DPPI*^{-/-} vehicle control, (D) *DPPI*^{-/-} TxA. Note the normal villous structure in control groups. In *DPPI*^{+/+} mice, note loss of villi, infiltration of granulocytes, and severe necrosis (*arrows*), pathologic effects that were diminished in *DPPI*^{-/-} mice. Scale bar = 100 μ m.

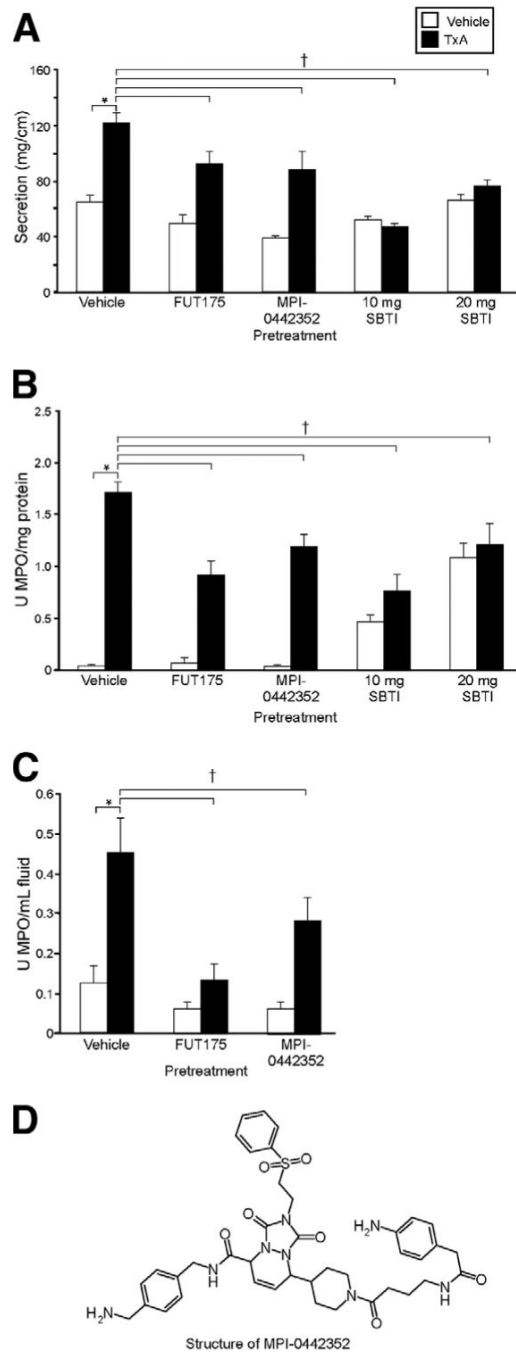


Figure 5.

Effect of pretreatment with trypsin and tryptase inhibitors on TxA-induced enteritis. Mice were preinjected with FUT-175, MPI-0442352, or SBTI before injection with vehicle (vehicle control, *open bars*) or 1.0 μ g TxA (*closed bars*). (A) Intestinal secretion expressed as milligram per centimeter of loop. (B) Tissue MPO expressed as units per milligram protein. (C) MPO in loop fluid expressed as units per milliliter of fluid. (D) Structure of MPI-0442352. * $P < .05$ between vehicle control and TxA loops in vehicle-pretreated mice. † $P < .05$ between results obtained with TxA in mice pretreated with vehicle compared with protease inhibitors.

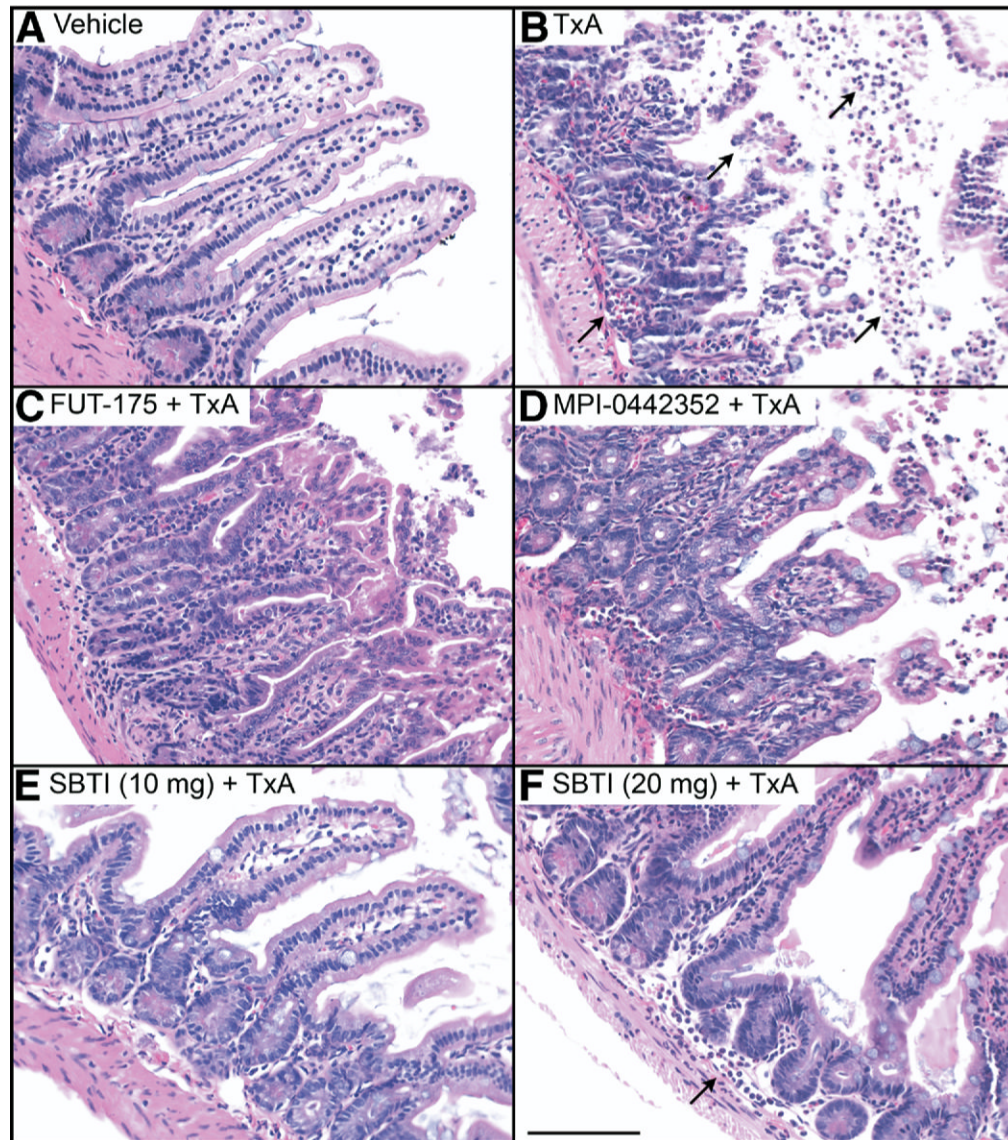


Figure 6. Effect of pretreatment with protease inhibitors on TxA-induced pathologic changes. Images show the ileum. (A) Vehicle control, (B) TxA, (C) FUT-175 plus TxA, (D) MPI-0442352 plus TxA, (E) SBTI (10 mg) plus TxA, (F) SBTI (20 mg) plus TxA. TxA caused a marked loss of villi, edema, neutrophil infiltration, and necrosis (*arrows*). SBTI pretreatment decreased epithelial damage and edema, but neutrophil infiltration was sometimes prominent (*arrow*). Scale bar = 100 μ m.

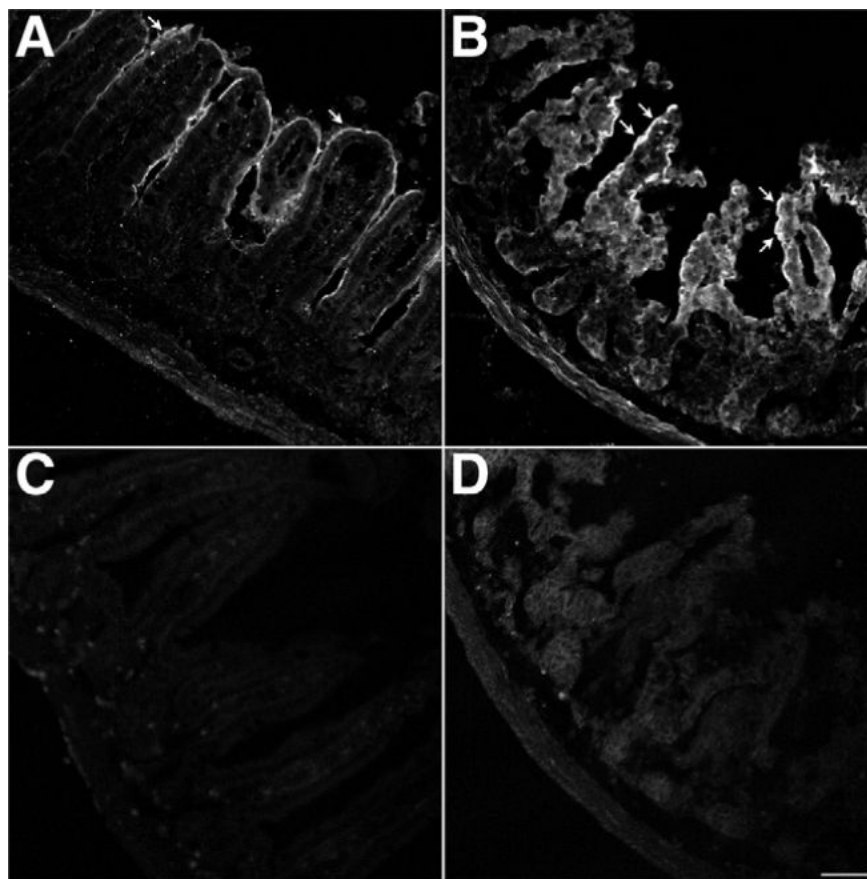
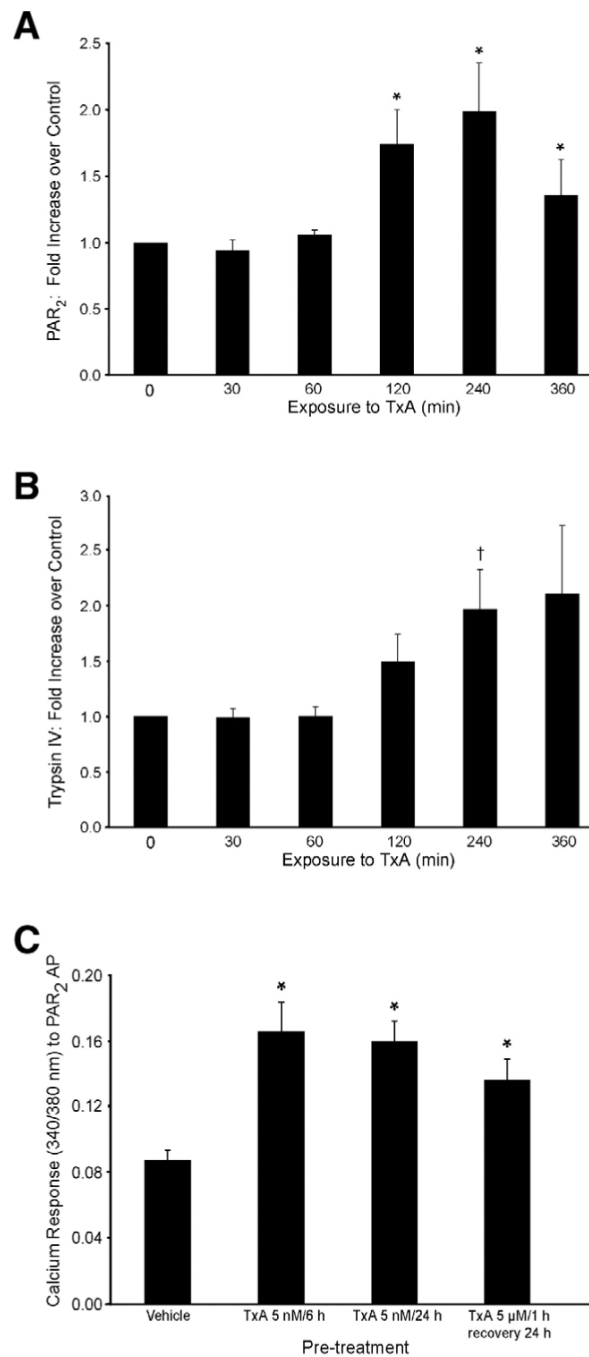


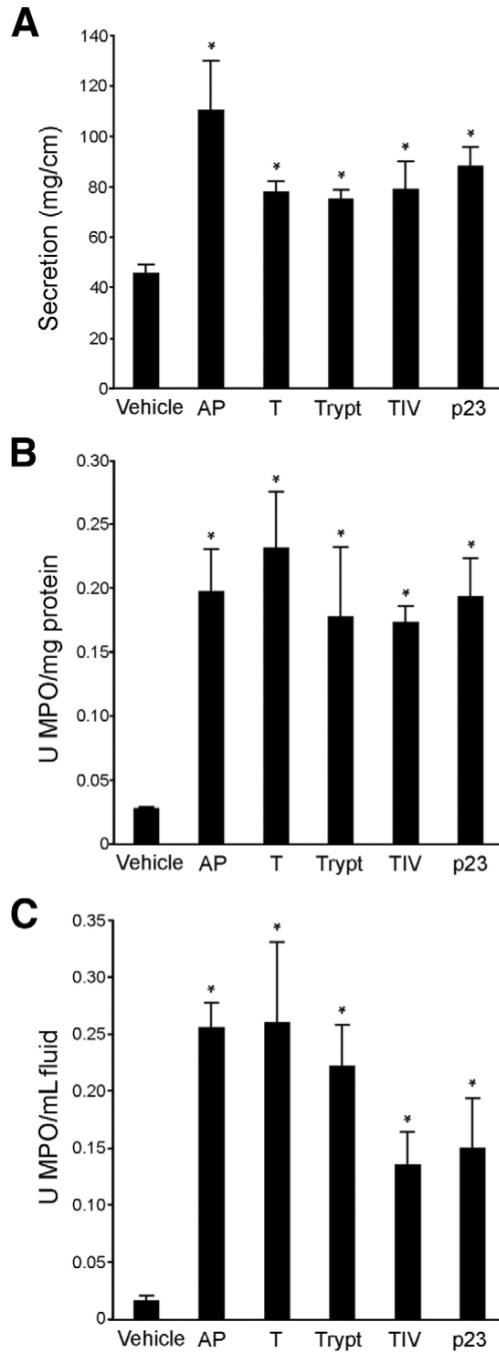
Figure 7.

Effects of TxA on PAR₂ expression. Ileal loops formed in wild-type mice were injected with (A and C) vehicle or (B and D) 1.0 μ g TxA. Tissue was stained for PAR₂ using a mouse anti-PAR₂ antibody 9717 and (A and B) a goat anti-rabbit secondary antibody conjugated to FITC or (C and D) the goat anti-rabbit secondary antibody conjugated to FITC alone. Images were acquired by confocal microscopy and are unmodified. The images shown in A and B were acquired under identical conditions to optimize the quantification of pixel intensity above baseline. The images shown in C and D were acquired under identical conditions to optimize tissue visualization. (A) Basal PAR₂ expression with positive enterocytes (*arrow*). (B) TxA markedly up-regulated PAR₂ expression in enterocytes (*arrows*). Omission of the mouse anti-PAR₂ antibody 9717 eliminated specific staining in both (C) vehicle-treated ileum and (D) TxA-treated ileum. *Scale bar* = 50 μ m.

**Figure 8.**

Effects of TxA on NCM460 cells. (A) Real-time quantitative PCR of PAR₂ levels. Results of 3 experiments are shown. After 120 minutes of exposure to TxA, PAR₂ levels were increased and remained increased even after 360 minutes. (B) Real-time quantitative PCR of trypsin IV levels. Results of 3 experiments are shown. After 120 minutes of exposure to TxA, trypsin IV levels were increased and remained increased even after 360 minutes. (C) Ca²⁺ mobilization in response to PAR₂ AP or vehicle (1/1300 dilution of 0.05 mol/L Tris in NCM culture medium) in cells pretreated with 5 nmol/L or 5 μmol/L TxA. Ca²⁺ responses to PAR₂-AP (10⁻⁵ mol/L) are expressed as a 340/380 ratio (peak baseline; n = 38–45 cells). TxA significantly enhanced

responses to PAR₂-AP. * $P < .05$ between control or 0 minutes and TxA-exposed cells. † $P = .055$ between control or 0 minutes and TxA-exposed cells.

**Figure 9.**

Effect of activation of PAR₂ on inflammation in the ileum. Mouse ileum was injected with AP (SLIGRL), pancreatic trypsin (T), trypsin (Trypt), trypsin IV (TIV), p23, or vehicle. Inflammation was assessed as before (Figures 1, 3, and 5). (A) All agents increased intestinal secretion, expressed as milligrams per centimeter of loop, with AP having the largest effect. (B) All agents increased tissue MPO, expressed as units MPO per milligram protein, to similar levels. (C) Fluid MPO, expressed as units MPO per milliliter fluid, was most strikingly increased by AP, trypsin, and trypsin. * $P < .05$ between control and results obtained by activating agents.

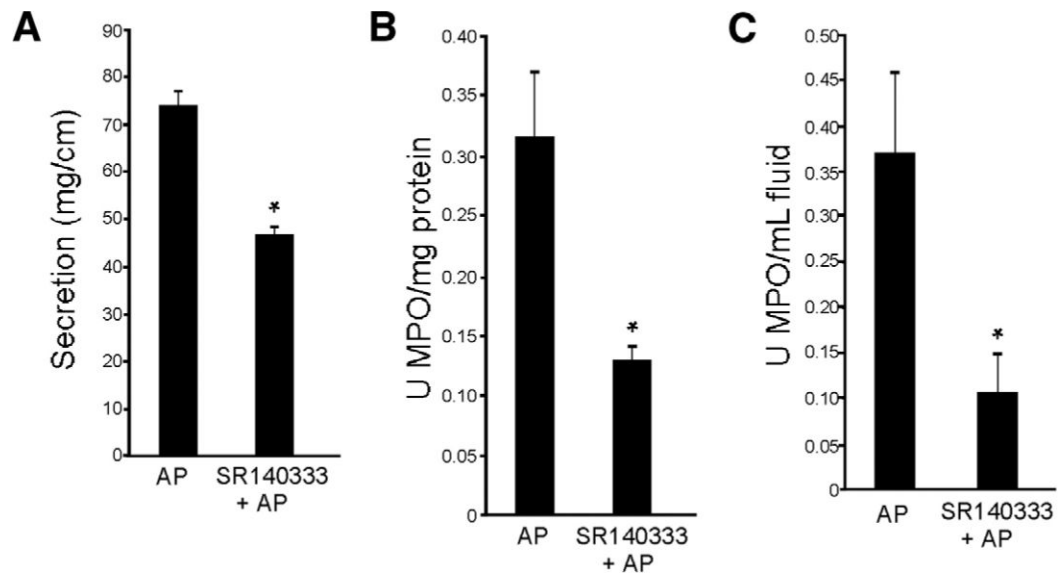


Figure 10.

Effect of NK1R inhibition on PAR₂-induced inflammation in the ileum. Mouse ileum was injected with carrier (1% dimethyl sulfoxide) or SR140333 60 minutes before AP. (A) Intestinal secretion, expressed as milligram per centimeter of loop, (B) tissue MPO, expressed as units MPO per milligram protein, and (C) fluid MPO, expressed as units MPO per milliliter fluid, were all significantly decreased by pretreatment with SR140333. * $P < .05$ between AP and results obtained by pretreatment with SR140333 followed by AP.

Table 1
 Histologic Score for TxA-Induced Enteritis in $Par_2^{+/+}$, $Par_2^{+/-}$, and $Par_2^{-/-}$ Mice

Mice genotype and treatment	Epithelial damage	Edema congestion	Polymorphonuclear leukocyte infiltration	Total score
$Par_2^{+/+}$ + vehicle control	0.16 ± 0.1	0.50 ± 0.1	0.68 ± 0.3	1.34 ± 0.4
$Par_2^{+/+}$ + TxA	0.91 ± 0.1 ^a	1.35 ± 0.1 ^a	2.21 ± 0.1 ^a	4.47 ± 0.3 ^a
$Par_2^{+/-}$ + vehicle control	0.06 ± 0.1	0.25 ± 0.1	0.81 ± 0.1	1.13 ± 0.2
$Par_2^{+/-}$ + TxA	0.89 ± 0.2	1.00 ± 0.1	1.73 ± 0.2 ^b	3.61 ± 0.4
$Par_2^{-/-}$ + vehicle control	0.35 ± 0.2	0.45 ± 0.2	0.75 ± 0.1	1.55 ± 0.5
$Par_2^{-/-}$ + TxA	0.25 ± 0.1 ^b	0.75 ± 0.2 ^b	1.70 ± 0.2 ^b	2.70 ± 0.3 ^b

^a $P < .05$ compared with $Par_2^{+/+}$ vehicle control group.

^b $P < .05$ compared with $Par_2^{+/+}$ TxA group.

Table 2
 Histologic Score for TxA-Induced Enteritis in *DPPI^{+/+}* and *DPPI^{-/-}* Mice

Mice genotype and treatment	Epithelial damage	Edema congestion	Polymorphonuclear leukocyte infiltration	Total score
<i>DPPI^{+/+}</i> + vehicle control	0.18 ± 0.1	1.28 ± 0.2	0.90 ± 0.2	2.35 ± 0.4
<i>DPPI^{+/+}</i> + TxA	1.64 ± 0.2 ^a	2.13 ± 0.2 ^a	2.38 ± 0.2 ^a	6.08 ± 0.5 ^a
<i>DPPI^{-/-}</i> + vehicle control	0.16 ± 0.1	0.64 ± 0.1	0.73 ± 0.1	1.52 ± 0.2
<i>DPPI^{-/-}</i> + TxA	1.32 ± 0.1	1.65 ± 0.1 ^b	2.32 ± 0.1	5.38 ± 0.3

^a $P < .05$ compared with *DPPI^{+/+}* vehicle control group.

^b $P < .05$ compared with *DPPI^{+/+}* TxA group.

Table 3
Histologic Score for TxA-Induced Enteritis in Mice Pretreated With Protease Inhibitors

Pretreatment + treatment	Epithelial damage	Edema congestion	Polymorphonuclear leukocyte infiltration	Total score
Vehicle + vehicle control	0.44 ± 0.1	0.97 ± 0.1	1.29 ± 0.2	2.73 ± 0.4
Vehicle + TxA	1.61 ± 0.1 ^a	1.77 ± 0.1 ^a	1.90 ± 0.1	5.21 ± 0.2 ^a
FUT-175 + vehicle control	1.02 ± 0.1	0.02 ± 0.1	0.88 ± 0.1	2.65 ± 0.2
FUT-175 + TxA	1.03 ± 0.2 ^a	1.51 ± 0.2	1.77 ± 0.2	4.35 ± 0.3 ^a
MPI-0442352 + vehicle control	0.56 ± 0.1	0.48 ± 0.02	0.56 ± 0.1	1.60 ± 0.2
MPI-0442352 + TxA	1.67 ± 0.2	1.93 ± 0.2	1.98 ± 0.2	5.55 ± 0.5
10 mg SBTI + vehicle control	0.30 ± 0.1	1.30 ± 0.1	1.46 ± 0.1	3.07 ± 0.2
10 mg SBTI + TxA	0.53 ± 0.1 ^b	1.17 ± 0.3 ^b	1.63 ± 0.1	3.32 ± 0.3 ^b
20 mg SBTI + vehicle control	0.43 ± 0.1	0.56 ± 0.1	0.79 ± 0.1	1.87 ± 0.2
20 mg SBTI + TxA	0.86 ± 0.1 ^b	1.06 ± 0.1 ^b	1.24 ± 0.1	3.13 ± 0.3 ^b

NOTE. Vehicle refers to vehicle for protease inhibitors, and vehicle control refers to vehicle for TxA.

^a*P* < .05 compared with inhibitor vehicle + vehicle control group.

^b*P* < .05 compared with inhibitor vehicle + TxA group.