Loss of purinergic vascular regulation in the colon during colitis is associated with upregulation of CD39

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Neshat S, deVries M, Barajas-Espinosa AR, Skeith L, Chisholm SP, Lomax AE. Loss of purinergic vascular regulation in the colon during colitis is associated with upregulation of CD39. Am J Physiol Gastrointest Liver Physiol 296: G399–G405, 2009.—Evidence from patients with inflammatory bowel disease (IBD) and animal models suggests that inflammation alters blood flow to the mucosa, which precipitates mucosal barrier dysfunction. Impaired purinergic sympathetic regulation of submucosal arterioles, the resistance vessels of the splanchnic vasculature, is one of the defects identified during IBD and in mouse models of IBD. We hypothesized that this may be a consequence of upregulated catabolism of ATP during colitis. In vivo and in vitro microscopy techniques were employed to measure the effects of purinergic agonists and inhibitors of CD39, an enzyme responsible for extracellular ATP catabolism, on the diameter of colonic submucosal arterioles from control mice and mice with dextran sodium sulfate (DSS) colitis. Using a luciferase-based ATP assay, we examined the degradation of ATP and utilized real-time PCR, Western blotting, and immunohistochemistry to examine the expression and localization of CD39 during colitis. Arterioles from mice with DSS colitis did not constrict in response to ATP (10 μM) but did constrict in the presence of its nonhydrolyzable analog α,β-methylene ATP (1 μM). α,β-Methylene ADP (100 μM), an inhibitor of CD39, restored ATP-induced vasoconstriction in arterioles from mice with DSS-induced colitis. CD39 protein and mRNA expression was markedly increased during colitis. Immunohistochemical analysis demonstrated that, in addition to vascular CD39, F4/80-immunoreactive macrophages accounted for a large proportion of submucosal CD39 staining during colitis. These data implicate upregulation of CD39 in impaired sympathetic regulation of gastrointestinal blood flow during colitis.

purinergic neurotransmission; ectonucleotidase; inflammation; sympathetic; vasoconstriction

STUDIES OF PATIENTS WITH INFLAMMATORY bowel disease (IBD) and animal models of IBD have revealed alterations in gastrointestinal (GI) blood flow (2, 15, 22, 26) and angiogenesis (4–6). These changes may contribute to disease pathogenesis, inasmuch as defects in mucosal perfusion render the mucosal barrier susceptible to breakdown. One potential mechanism of altered mucosal blood flow is defective neural regulation of GI blood vessels during inflammation.

Submucosal arterioles are the resistance vessels of the splanchnic vasculature and thus regulate mucosal perfusion (13, 23, 31). We previously identified a defect in sympathetic vasoconstrictor regulation of colonic submucosal arterioles in the 2,4,6-trinitrobenzene sulfonic acid (TNBS) mouse model of IBD (22). Neurally evoked vasoconstrictions in control mice were sensitive to α1-adrenoceptor and purinergic receptor antagonism, and superfusion of ATP and phenylephrine caused robust vasoconstrictions. In contrast, arterioles from mice with colitis constricted to phenylephrine but not to ATP, and nerve-evoked constrictions were insensitive to purinergic receptor blockade. Importantly, a nonhydrolyzable analog of ATP, α,β-methylene ATP, constricted control and inflamed arterioles, suggesting that colitis upregulates catabolism of neurally released and exogenously applied ATP. Similar findings were subsequently reported in mesenteric vessels of patients with IBD (2). The aim of the present study was to determine the mechanism of this sympathetic neuropathy in the dextran sodium sulfate (DSS) mouse model of IBD.

Extracellular ATP binds two major classes of cell surface receptors: ligand-gated ion channels (P2X receptors) and G-protein-coupled (P2Y) receptors. The predominant effect of purinergic neurotransmitters on GI blood vessels is vasoconstriction due to activation of P2X receptors (20, 32). A diverse family of enzymes, including ectonucleoside 5’-triphosphohydrolases (ENTPDs, CD39 family), ectonucleotide pyrophosphatase/phosphodiesterases, and alkaline phosphatases, regulate the extracellular concentration of purines (33). The CD39 family hydrolyzes ATP and ADP to AMP, which in turn is hydrolyzed to adenosine by CD73. Since vascular tissue upregulates extracellular ATPase activity in response to hypoxia (9), we hypothesized that this process may underlie the defective purinergic vasoconstriction observed in the colon during colitis.

MATERIALS AND METHODS

Animals and materials. Male CD-1 mice (25–35 g body wt) were obtained from Charles River (Saint-Constant, QC, Canada). Experimental protocols were approved by the Queen’s University Animal Care Committee and conformed to the Guidelines of the Canadian Council of Animal Care. Animals had access to standard laboratory chow and tap water ad libitum. All reagents were bought from Sigma Aldrich (St. Louis, MO) unless otherwise stated.

DSS model of IBD. Colonic inflammation was induced by administration of 5% DSS (wt/vol; MP Biomedicals, Solon, OH) in drinking water for 5 days, followed by normal drinking water for 1–3 days. Control mice received normal drinking water for the 6- to 8-day study period. The DSS-treated mice were monitored daily for signs of distress, altered feeding/drinking habits, loose and/or bloody stool, and weight loss.

Assessment of inflammation. Segments of proximal colon from control and DSS-treated mice were dissected, flash frozen in an...
Eppendorf tube in liquid nitrogen, and stored at −80°C for 7–14 days until assayed for myeloperoxidase (MPO) activity, a measure of neutrophil infiltration, as previously described (24). Values are expressed as units of MPO activity per gram of tissue sample, where one unit of MPO is defined as that which degrades 1 μmol of hydrogen peroxide per minute.

**Intravital microscopy.** Mice were anesthetized with an intraperitoneal injection of ketamine hydrochloride (200 mg/kg; Rogar/STB, Montreal, QC, Canada) and xylazine (10 mg/kg; MTC Pharmaceuticals, Cambridge, ON, Canada). The right jugular vein was cannulated with a heparin-coated, 600-μm polyethylene catheter for further anesthetic administration, if required. The mice were positioned on a Plexiglas stage adaptor, a laparotomy was performed using a cautery, and the colon was carefully externalized using saline-moistened cotton swabs. The distal colon was cauterized along the antimesenteric border, opened along the cauterized line using iris scissors, clipped, and the preparations were washed three times in PBS before incubation in HEPES-buffered saline (in mM: 140 NaCl, 4.7 KCl, 20 NaHCO3, 1.2 MgSO4, and 2.0 CaCl2) for 1 h at room temperature. ATP standards and submucosal preparations were incubated in HEPES-buffered saline (in mM: 140 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 2 CaCl2, and 1 MgCl2 (pH 7.4)) at 37°C for 10 min after dissection. The tissue samples were then transferred into tubes containing 2 ml of 10 μM ATP and incubated for 15 min in a 37°C water bath. The samples and ATP standards were diluted 1:10 with chilled saline (900 μl of saline + 100 μl of sample) and immediately assayed for ATP concentration using a luminometer.

**ATP assay.** Extracellular ATP concentration was measured using a luciferase-based assay (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. For measurement of the amount of ATP degradation by colonic submucosa from control and DSS mice, ATP standards and submucosal preparations were incubated in HEPES-buffered saline in a water bath at 37°C. Preliminary experiments showed that these parameters evoked maximal TTX-sensitive vasoconstrictions. Immediately after experiments, mice were euthanized by intraperitoneal overdose of the ketamine-xylazine mixture and cervical dislocation. Constriction amplitudes are expressed as a percentage of resting diameter.

**In vitro video microscopy.** Mice were anesthetized by isoflurane inhalation and euthanized by cervical transection and exsanguination. Distal colon was removed and placed in Krebs solution (in mM: 126 NaCl, 2.5 NaH2PO4, 1.2 MgCl2, 2.5 CaCl2, 5 KCl, 25 NaHCO3, and 11 glucose) that had been bubbled with 95% O2-5% CO2 to maintain pH 7.4. Submucosal arterioles were localized using a long-working-distance (16 mm) × 25 objective lens. Experiments were recorded on a videocassette recorder (AG-3200 S-VHS, Panasonic, Mississauga, ON, Canada) for post hoc analysis with Image Pro Plus 5 software (Media Cybernetics, Silver Spring, MD). The chosen arterioles were allowed to stabilize for 20 min to ensure that inner vessel diameter did not change as a function of time. Adenosine 5'-triphosphate magnesium salt (ATP, 10 μM) was superfused over the viscera for 5 min, and the maximal constriction was measured. After a 10-min washout period, nerve-evoked vasoconstriction was measured during 30 s of electrical field stimulation (EFS; 20 Hz, 0.5-ms duration, 50–60 V; SD9 stimulator, Grass, Longueil, QC, Canada). Preliminary experiments showed that these parameters evoked maximal TTX-sensitive vasoconstrictions. Immediately after experiments, mice were euthanized by intraperitoneal overdose of the ketamine-xylazine mixture and cervical dislocation. Constriction amplitudes are expressed as a percentage of resting diameter.

**Real-time PCR.** RNA was extracted from ~100 mg of distal colon from control and DSS-treated mice with use of the RNaqueous kit (Ambion, Austin, TX). The RNA was reprecipitated using LiCl precipitation solution (Ambion). Subsequently, cDNA was reverse transcribed from 1 μg of total RNA using SuperScript III (Invitrogen) and oligo(dT)12-18 primers (Invitrogen). Real-time PCR was performed using the primers listed in Table 2 with a Roche Lightcycler and the Quantitech SYBR Green PCR kit (Qiagen). The target-to-reference ratio was then calculated using Realquant software (Roche).

**Immunohistochemistry.** Submucosal preparations from control or DSS colons were fixed overnight in 4% paraformaldehyde at 4°C. Tissues were washed three times for 10 min each in PBS and then incubated for 1 h in 10% normal horse serum in 1% Triton X-100 in PBS. Tissues were washed three times in PBS before incubation overnight in a primary antiserum (Table 1). Primary antiserum was removed, and the preparations were washed three times in PBS for 10 min and then incubated for 2 h in the appropriate secondary antiserum...
(Table 1). This process was repeated for the other primary antiserum, if necessary. After three final washes for 10 min each, preparations were mounted on slides in buffered glycerol, and coverslips were applied. Controls for antiserum specificity included preadsorption of the anti-F4/80 antiserum with blocking peptide. In the case of the CD39 antiserum, no blocking peptide was available, so mismatched combinations of primary and secondary antibodies were used as negative controls. In addition, the immunoblots with anti-CD39 antiserum yielded a single band of immunoreactivity at the expected molecular weight of membrane-bound CD39. The slides were then analyzed with an epifluorescence microscope (Olympus BX51). Images were acquired using a Coolsnap charge-coupled device camera (Photometrics, Tucson, AZ) and Image Pro software.

RESULTS

Mice that drank DSS-containing water for 5 days exhibited well-characterized symptoms of colitis, including weight loss and loose, bloody stool. MPO activity was significantly higher in colons from mice exposed to DSS for 5 days than untreated controls: 7.04 ± 0.6 (n = 34 mice) vs. 0.88 ± 0.1 (n = 22 mice) units/mg tissue (P < 0.001, unpaired t-test with Welch’s correction).

In vivo and in vitro constrictions to purinergic receptor agonists. Electrical field stimulation (EFS) of the splanchnic nerves in vivo caused large vasoconstrictions in submucosal arterioles from control mice. However, the amplitude of EFS-evoked constrictions was significantly reduced during colitis (Fig. 1A). Submucosal arterioles from control animals always constricted to 10 μM ATP superfused over the visceras in vivo (Fig. 1B), whereas vessels from mice with colitis did not. In vitro measurements of submucosal arteriole constriction revealed that arterioles from animals with DSS colitis did not constrict to ATP but did constrict to a nonhydrolyzable analog, α,β-methylene ATP (1 μM; Fig. 1C). These findings are in agreement with a previous study of the effects of TNBS-induced colitis on purinergic vasoconstriction (22) and suggest that upregulated hydrolysis of extracellular ATP underlies the decrease in sympathetic vasoregulation during colitis.

To examine whether the defect in purinergic vascular regulation is due to loss of ligand, rather than receptor, we made immunoblots from submucosal preparations of control mice and mice with colitis. Western blotting for the P2X1 receptor, which underlies purinergic vasoconstriction (20, 32), revealed that colitis did not reduce receptor expression in the colonic submucosa (Fig. 2; n = 4 each). These data, along with data shown in Fig. 1C, indicate that the loss of purinergic vasoconstriction is not due to loss of P2X1 receptors and, therefore, may be due to upregulation of enzymes that hydrolyze ATP. The remainder of this study was devoted to identifying the enzyme that underlies the enhanced degradation of ATP during colitis.

Effects of colitis on nucleotidases. Extracellular ATP can be hydrolyzed by membrane-bound or soluble ATPases (33). We therefore examined whether enhanced activity of soluble ectonucleotidases was responsible for the absence of purinergic vasoconstriction during colitis. No significant difference was detected in the amount of ATP hydrolysis between colonic tissue from control mice and mice with colitis: 8.6 ± 4% (n = 6) and 9.8 ± 3% (n = 11) breakdown, respectively (P = 0.8, Mann-Whitney test). This indicates that enhanced soluble ectonucleotidase activity does not underlie purinergic signaling deficits during colitis, implicating membrane-bound ectonucleotidase activity in this deficit.

Among membrane-bound ectonucleotidases, CD39, also known as ENTPD1, is upregulated in the vasculature during ischemia (9, 30). Therefore, we utilized pharmacological agents to examine its role in colitis. Submucosal preparations from control mice or mice with colitis were examined for responsiveness to ATP (10 μM) before and after incubation in the competitive inhibitor of CD39, α,β-methylene ADP (100 μM). Arterioles from mice with colitis did not constrict to ATP but did constrict to phenylephrine (3 μM; Fig. 3B). Preincubation of arterioles from animals with colitis, but not controls, in α,β-methylene ADP significantly increased the amplitude of constrictions to ATP (P < 0.001, 1-way ANOVA with Bonferroni’s multiple comparison test; Fig. 3A vs. Fig. 3B). This suggests that colitis upregulated CD39 activity. A different CD39 antagonist, ARL-67156 (100 μM), was also examined to determine whether it could recover responsiveness to ATP in arterioles from mice with colitis. In contrast to α,β-methylene ADP, it did not significantly recover arteriolar constrictions to ATP: 1 ± 1% constriction to ATP and 7.1 ± 3% constriction to ATP following preincubation in ARL-67156 (P = 0.09, Mann-Whitney test, n = 8 each). In a recent study of the effect of ARL-67156 on recombinant mouse and human CD39 family members, Levesque et al. (21) reported that mouse CD39 is only 50% inhibited by ARL-67156 when the ratio of antagonist to ATP is 10:1. This may account for the relatively small recovery of constrictions to ATP after ARL-67156 treatment.

Expression of CD39 during colitis. Western blots were used to examine whether the expression of CD39 was altered by colitis. Immunoblots for CD39 of submucosal preparations and whole-thickness preparations from colons of control mice and mice with colitis were analyzed and compared with β-actin as a protein-loading control. Densitometry analysis of CD39 and β-actin revealed that colitis significantly increased CD39 expression in the whole colon and the submucosa (Fig. 4). The antisera used for CD39 immunoblotting is immunoreactive with a residue present on several isoforms of ENTPD. Therefore, we used real-time PCR to determine which subtypes of ENTPD are expressed in the mouse colon and whether colitis

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ENTPD, ectonucleotidase 5’-triphosphate diphosphohydrolase.
altered their expression. ENTPD2 expression was not detected in normal or inflamed mouse colon (data not shown) and was not considered further. ENTPD1 and ENTPD3 were expressed in the colon, and real-time PCR indicated that ENTPD1, but not ENTPD3, was upregulated during colitis (Fig. 4). These findings led us to determine the immunohistochemical localization of CD39 in the submucosa in colons from control mice and those with colitis (Fig. 5). In control animals, CD39 was prominently expressed on submucosal arterioles (Fig. 5A, representative of preparations from 6 animals). During inflammation, vascular expression was slightly increased, and there was a dramatic increase in extravascular immunoreactivity for CD39 (Fig. 5B, representative of preparations from 6 animals). We double labeled preparations from control mice and mice with colitis with the macrophage marker F4/80 to determine whether macrophages contribute to the increase in extravascular CD39 immunoreactivity during colitis. As shown in Fig. 5, C–F (representative of preparations from 4 mice with colitis), many of the nonvascular cells that express CD39 during colitis were F4/80-immunoreactive macrophages.

**DISCUSSION**

Studies in mesenteric arteries from patients with IBD (2), submucosal arterioles from mice with TNBS colitis (22), and our present study of DSS colitis have each demonstrated that colitis impairs purinergic vasoregulation in the GI tract. These findings highlight the importance of determining the mechanism of altered purinergic vasoregulation during intestinal inflammation. The main findings of the present investigation are that CD39, an enzyme that hydrolyzes ATP and ADP, is upregulated during colitis and that inhibition of this enzyme restores arteriolar constrictions to ATP. Since sympathetic regulation of GI blood flow is compromised during colitis, inhibition of CD39 may restore normal mucosal perfusion and aid in the restoration of mucosal barrier function.
ATP as a sympathetic vasoconstrictor. ATP has been shown to be a sympathetic vasoconstrictor in many different vascular beds in a variety of species, including human, rabbit, and mouse mesenteric arteries (2, 28, 32), rabbit ear arteries (17), rat tail (29) and femoral arteries (16), and guinea pig submucosal arterioles (10). ATP released from sympathetic nerve terminals in vascular smooth muscle binds P2X1 receptors on myocytes and causes vasoconstriction, whereas mechanically induced ATP release from endothelial cells acts on endothelial P2 receptors and leads to vasodilation (3, 14, 20, 32). In the present study, we examined the vasoconstrictor effects of ATP and found that inflammation impedes ATP-induced vasoconstriction. Although it was not examined in the present study, it is likely that endothelium-dependent vasodilation is also compromised during inflammation. If upregulation of vascular CD39 expression is a common response to inflammation, it could contribute to alterations in blood flow in other tissues during inflammation.

Ectonucleotidases and inflammation. Because of the prominent role of ATP as an intercellular signaling molecule, a family of enzymes known as ectonucleotidases have been identified that can degrade ATP to AMP and adenosine. In addition to their role in maintaining extracellular nucleotide levels, ectonucleotidases also play a role in the regulation of inflammation.

Fig. 3. Inhibition of arteriolar constrictions to ATP during colitis is reversed after superfusion with a CD39 inhibitor. A: constrictions of control submucosal arterioles to phenylephrine, ATP, the CD39 antagonist α,β-methylene ADP, and ATP + α,β-methylene ADP. α,β-Methylene ADP had no effect on constrictions to ATP in control arterioles. B: constrictions to ATP were restored in arterioles from mice with DSS-induced colitis after superfusion with α,β-methylene ADP (n = 5 for each treatment for control mice and n = 6 for each treatment for DSS mice). *P < 0.001 (1-way ANOVA with Bonferroni’s multiple comparisons test). Response to α,β-methylene ADP was significantly (P < 0.001 by 1-way ANOVA with Bonferroni’s multiple comparisons test) smaller than constrictions to any other agonist, except ATP in DSS-treated mice.

ATP as a sympathetic vasoconstrictor. ATP has been shown to be a sympathetic vasoconstrictor in many different vascular beds in a variety of species, including human, rabbit, and mouse mesenteric arteries (2, 28, 32), rabbit ear arteries (17),

Fig. 4. Colitis increases whole-thickness colon and submucosal expression of CD39. A: Western blots of CD39 and β-actin from submucosa. B: densitometric quantification of CD39 expression relative to β-actin in whole-thickness and submucosal preparations from control and DSS-treated colons (n = 8 each). *P < 0.01 (Mann-Whitney test). C: real-time PCR analysis of ectonucleotidase 5’-triphosphate diphosphohydrolase (ENTPD) in whole-thickness colon samples from control mice and mice with DSS-induced colitis (n = 4 each). Colitis significantly increased ENTPD1, but not ENTPD3, expression. *P < 0.05 (Mann-Whitney test).
of enzymes that tightly regulate extracellular ATP concentration and, thus, access to and activation of purinergic receptors has evolved. Members of the CD39 family of enzymes are found in many cell types, including vascular endothelium, smooth muscle, lymphocytes, macrophages, platelets, and astrocytes (1, 11, 25, 34). They play diverse roles in intercellular signaling, regulating thrombus formation, T cell activation, vascular permeability, and glial Ca\textsuperscript{2+} wave propagation. The CD39 family is also important in modulating neuronal purinergic signaling. For example, inhibition of soluble ectonucleotidase activity increased the amplitude of purinergic responses to nerve stimulation in the vas deferens and enhanced the frequency of spontaneous excitatory junction potentials (12), suggesting a role for CD39 in moment-to-moment regulation of purinergic excitatory junction potentials in this tissue.

Inflammation is associated with tissue damage and increased release of adenine nucleotides from damaged cells. The CD39 family and CD73 participate in an adaptive response to the increase in extracellular nucleotide concentration by increasing their expression and activity to decrease ATP and increase adenosine (7). Increased CD39 expression has been reported in coronary vasculature, lungs, and pancreas during inflammation and tissue damage (8, 9, 19). Transgenic mice that lack ENTPD1 develop increased pulmonary edema and inflammation compared with control littermates in a ventilator-induced model of lung injury (8). In the cerulein model of pancreatitis, mice that lack ENTPD1 exhibited less severe atrophy and fibrosis, suggesting that upregulation of ENTPD1 is not protective in this tissue (19). It therefore appears that inhibition of extracellular ATP catabolism can have positive or negative effects, depending on the nature of inflammation. However, no one has considered the consequence of upregulated ATP catabolism for purinergic neurotransmission during inflammation.

**Identity of the CD39 subtype responsible for vascular dysregulation during colitis.** The members of the CD39/ENTPD family have different affinities toward ATP and ADP (33). ENTPD1 (CD39) binds ATP and ADP with almost equal affinity (1:0.8), whereas ENTPD2 (CD39L1) and ENTPD3 (CD39L3) have much higher affinities for ATP than ADP (1:0.03 vs. 1:0.3). The present study utilized the nonhydrolyzable ADP analog α,β-methylene ADP to inhibit the catalytic site of CD39. Our finding that α,β-methylene ADP recovered arteriolar responsiveness to ATP during colitis sug-

![Fig. 5. Immunohistochemical localization of CD39 in colonic submucosa. A: immunohistochemical detection of submucosal CD39 from colon of a control mouse. Note distinct immunoreactivity within a submucosal arteriole, along with occasional immunoreactive extravascular cells. B: during colitis, CD39 immunoreactivity is increased, and number of immunoreactive cells surrounding arterioles is dramatically increased. C and D: double-label immunohistochemistry identifies F4/80-immunoreactive macrophages as a major source of extravascular CD39 immunoreactivity during colitis. Note autofluorescence of the blood vessel in D due to the wavelength (350 nm) used to excite the anti-goat secondary antibody. E and F: higher-magnification micrographs of areas enclosed in rectangles in C and D, respectively. Note extent of colocalization between CD39- and F4/80-immunoreactive macrophages. Arrows, colocalization.](image-url)
gests that the enzyme subtype that contributes to loss of purinergic vasoregulation binds ADP with high affinity, implicating ENTPD1/CD39. Real-time PCR analysis of ENTPD 1, ENTPD2, and ENTPD3 mRNA expression in colons of control mice and mice with colitis supports this conclusion, inasmuch as ENTPD 1 but not ENTPD2 or ENTPD3, was increased during colitis.

Conclusions. It has become increasingly evident that extracellular purinergic signaling is disordered during disease and may play a role in pathogenesis. In the present study, we have identified an important role for ENTPD1 in defective vascular purinergic signaling is disordered during disease and may contribute to abnormal blood flow in vessels that utilize purines to regulate vascular blood flow. This may delay the restoration of mucosal barrier function and predispose the GI tract to relapsing and remitting inflammation.

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