Acute murine colitis reduces colonic 5-aminosalicylic acid metabolism by regulation of N-acetyltransferase-2

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Ramírez-Alcántara V, Montrose MH. Acute murine colitis reduces colonic 5-aminosalicylic acid metabolism by regulation of N-acetyltransferase-2. Am J Physiol Gastrointest Liver Physiol 306: G1002–G1010, 2014. First published April 17, 2014; doi:10.1152/ajpgi.00389.2013.—Pharmacotherapy based on 5-aminosalicylic acid (5-ASA) is a preferred treatment for ulcerative colitis, but variable patient response to this therapy is observed. Inflammation can affect therapeutic outcomes by regulating the expression and activity of drug-metabolizing enzymes; its effect on 5-ASA metabolism by the colonic arylamine N-acetyltransferase (NAT) enzyme isoforms is not firmly established. We examined if inflammation affects the capacity for colonic 5-ASA metabolism and NAT enzyme expression. 5-ASA metabolism by colonic mucosal homogenates was directly measured with a novel fluorimetric rate assay. 5-ASA metabolism reported by the assay was dependent on Ac-CoA, inhibited by alternative NAT substrates (iso-niazid, p-aminobenzoylglutamate), and saturable with $K_m$ (5-ASA) = 5.8 μM. A mouse model of acute dextran sulfate sodium (DSS) colitis caused pronounced inflammation in central and distal colon, and modest inflammation of proximal colon, defined by myeloperoxidase activity and histology. DSS colitis reduced capacity for 5-ASA metabolism in central and distal colon segments by 52 and 51%, respectively. Use of selective substrates of NAT isoforms to inhibit 5-ASA metabolism suggested that mNAT2 mediated 5-ASA metabolism in normal and colitis conditions. Western blot and real-time RT-PCR identified that proximal and distal mucosa had a decreased mNAT2 protein-to-mRNA ratio after DSS. In conclusion, an acute colonic inflammation impairs the expression and function of mNAT2 enzyme, thereby diminishing the capacity for 5-ASA metabolism by colonic mucosa.

Drug metabolism; fluorescence; dextran sulfate sodium; myeloperoxidase; kinetics; enzymatic assay; inflammatory bowel disease; enzyme isoform

THE TWO MAJOR INFLAMMATORY bowel diseases (IBD), ulcerative colitis (UC) and Crohn’s disease, afflict 1.4 million individuals in the United States (USA) and 2.2 million in Europe (42). To induce and maintain mucosal healing of the colonic mucosa, the major goal of UC therapy is to reduce or eliminate colonic inflammation. For mild to moderate inflammation, therapy based on the nonsteroidal anti-inflammatory compound 5-aminosalicylic acid (5-ASA) is frequently prescribed. In information compiled by the National Institutes of Health from the 2004 Verispan database of prescriptions filled at retail pharmacies in the USA, 5-ASA or its prodrugs accounted for 44% of Crohn’s disease prescriptions and 81% of UC prescriptions (23). It is not well understood how 5-ASA induces its anti-inflammatory effect on colonic mucosa, but multiple mechanisms have been proposed, including inhibition of intestinal macrophage chemotaxis (47), inhibition of proinflammatory cytokine release (25), inhibition of cyclooxygenase and prostaglandin pathways (28), inhibition of nuclear factor-κB activation (20), agonist of the peroxisome proliferator-activated receptor-γ (55), upregulation of the heat shock protein (heme oxygenase-1 enzyme) expression and activity (9, 22, 33), and antioxidative and free radical scavenger action (3, 53, 58). Although 5-ASA therapy is well tolerated and effective in patients with mild to moderate inflammation, many patients are refractory to this therapy or undergo relapse when 5-ASA is used to maintain remission (24). For this reason, and because of the undesirability of long-term corticosteroid use as the next level of UC therapy (46), there is strong interest in understanding the basis for variable outcomes with 5-ASA.

A long-standing hypothesis is that variable therapeutic outcomes are the result of variable 5-ASA metabolism among patients. After 5-ASA administration, the N-acetylation of 5-ASA produces the major metabolite (Ac-5-ASA), which can be detected in colonic tissue, colonic lumen, plasma, and urine along with minor metabolites (2, 19, 60). 5-ASA is known to act locally in the intestinal mucosa, since its efficacy is more closely correlated to drug mucosal concentration rather than blood concentration (17). This has focused attention on 5-ASA metabolism by N-acetyltransferase (NAT) enzymes in colonic mucosa.

Arylamine N-acetyltransferases are phase II xenobiotic metabolizing enzymes that catalyze the transfer of an acetyl moiety from acetyl-coenzyme A (Ac-CoA), or another acetyl donor, to the nitrogen (N-acetylation) or oxygen atom of numerous aromatic and heterocyclic amine drugs such as the antihypertensive hydralazine, antitubercular isoniazid, and antibacterial sulfonamides (23, 30, 52). NATs have been identified in numerous species, including humans (7) and mice (39) where two functional NAT isoforms have been described. Human NAT-1 (hNAT1) is the ortholog of mouse NAT-2 (mNAT2); and human NAT-2 (hNAT2) is the ortholog of mouse NAT-1 (mNAT1) (57). A third NAT has also been identified. In humans, it is a pseudogene (NATP) (7) and in mice an expressed but relatively inactive mNAT3 isoform (22). There is functional homology between the human and mouse orthologs of isoforms 1 and 2 at the level of tissue distribution, developmental expression, substrate specificity, sequence similarity, and molecular structure (57). The hNAT1/mNAT2 isoform is expressed in a wide range of tissues, including liver, colon, small intestine, placenta, lungs, kidney, bladder, blood, and skin (11, 18, 32, 59). In contrast, hNAT2/mNAT1 is expressed mainly in liver, small intestine, and colon (13, 14, 29, 32, 35).

It remains an open question how 5-ASA metabolism affects drug efficacy against colitis. It has been difficult to link the outcomes of 5-ASA therapy to NAT gene polymorphisms that produce rapid or slow acetylation (27, 31, 54) because the
human and mouse colon express both NAT isoforms (11, 18, 32, 64), and 5-ASA is a substrate for both NAT isoforms (22, 38). It also remains unclear whether 5-ASA or its metabolite is an active moiety of therapy. In both cell-free and intact-cell assays, 5-ASA is more potent as an antioxidant than Ac-5-ASA (1, 4, 26, 58), but the two compounds have been reported to have similar potency at inhibiting lipooxygenase (5), eicosanoid production (21), interferon-γ action (13, 14), lipid peroxidation (67), and scavenging HOCl (63). While topical application of Ac-5-ASA to the colon may be of limited or no therapeutic value (61, 66), negative findings may be explained by the slow uptake of Ac-5-ASA vs. 5-ASA by colonocytes (37). Conceptually, unless both 5-ASA and Ac-5-ASA are equally effective as anti-inflammatory drugs, changes in NAT activity are predicted to change the efficacy of 5-ASA therapy.

Inflammation is known to change activity of many enzymes and transporters that affect therapeutic drugs (8, 45, 51, 62, 65); therefore, for drug efficacy, it is important to know if this central feature of IBD is by itself affecting 5-ASA metabolism. Unfortunately, this has only been examined in small studies using IBD patients with diverse clinical status, and results are conflicting. It has been reported that IBD patients with active disease have a lowered plasma Ac-5-ASA-to-5-ASA ratio vs. healthy controls (19), but this conflicts with observations of others who saw no change in 5-ASA acetylation rates in colonic biopsy homogenates or isolated colonocytes from IBD patients (2, 36, 41). At present, we cannot firmly predict if decreased 5-ASA metabolism is a reproducible finding in inflamed tissue or if such a change in metabolism would improve or reduce the clinical benefit of 5-ASA therapy. In this study, we approach the first aspect of this uncertainty by validating a fluorimetric assay to directly measure Ac-5-ASA production and then use a murine acute colitis model to test if inflammation modulates colonic 5-ASA metabolic capacity and regulates expression of colonic NAT enzyme.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were housed and bred in facilities with controlled room temperature (70 ± 2°F), 30–70% humidity, and 14:10-h light-dark cycles. Mice were fed with standard pellet diet and tap water ad libitum. All experiments and protocols for animal use were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Dextran sulfate sodium-induced colitis. Twelve-week-old mice received 2.5% wt/vol dextran sulfate sodium (DSS) (molecular weight 36–50 kDa; MP Biomedical, Solon, OH) dissolved in water for 5 days, then switched to regular drinking water for a subsequent 2 days, and killed. Age-matched control mice received regular water.

Colonic tissue preparation. After animal death, the entire colon was removed and placed immediately in ice-cold PBS (pH 7.4), and then colon length was measured. Luminal contents were flushed out with cold PBS, and the gut was slit open longitudinally on the antimesenteric side. For the colitis study, the entire length of colon was then divided in half longitudinally, with one half used for histology and the other half used for biochemical analyses. For each use, colon tissue was divided into the following three sections: the area including all macroscopically visible rugae was designated as central and distal colon (see Fig. 5A).

Histology. Full-thickness colonic tissue was staged as a Swiss roll (44), fixed in 10% neutral buffered formalin for 24 h, transferred to 70% ethanol, and embedded in Paraplast (Fisher Scientific, Pittsburgh, PA). Tissue sections (4 µm) were stained with hematoxylin and eosin and subjected to blinded evaluation by a pathologist.

Homogenate for biochemical analyses. Colonic tissue was muscle stripped. Resultant mucosal sheets (in some cases divided into proximal, central, and distal sections as described) were homogenized in 300–500 µl of ice-cold Sorensen’s sodium phosphate buffer (0.1 M, pH = 7.0) with 1 mM EDTA, 1 mM DTT, and protease inhibitors (mini Complete; Roche Applied Science, Indianapolis, IN). An aliquot of homogenate was prepared for myeloperoxidase (MPO) activity measurement as described below, remaining homogenate was centrifuged at 1,000 g for 3 min at 4°C, and supernatants were separated into aliquots and stored at −80°C until use.

MPO activity. MPO activity in colon mucosal homogenates was determined colorimetrically (40). Mucosa homogenate was mixed with 50 mM potassium phosphate buffer (pH = 6) and a final concentration of 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich, St. Louis, MO). Homogenates were sonicated on ice for 15 s, subjected to three freeze-thaw cycles (liquid nitrogen-37°C water bath), and then centrifuged at 20,000 g for 30 min at 4°C. MPO activity of supernatant was assayed at room temperature in 50 mM potassium phosphate buffer (pH = 6) containing 0.5 mM o-dianisidine hydrochloride (Sigma-Aldrich) and 0.0005% H2O2 (Fisher Scientific). Absorbance at 450 nm was recorded for the initial 2 min of the enzymatic reaction. MPO specific activity was calculated from rates of absorbance change, normalized to protein amount (bicinchoninic acid method; Pierce Biotechnology, Rockford, IL), and assuming a molar extinction coefficient of 1.13 × 10−2 µmol·cm of oxidized o-dianisidine.

Assay of 5-ASA metabolism by NAT. For routine assay, colon mucosal homogenate was diluted to 0.25–0.50 mg/ml in Sorensen’s sodium phosphate buffer (0.1 M, pH = 7.0) and mixed with the NAT cofactor Ac-CoA (0.420 mM; Sigma-Aldrich). After equilibration 5 min at 30°C, 5-ASA (25 µM; Sigma-Aldrich) was added to start the reaction. Rate of Ac-5-ASA production was measured for the initial 2 min of the enzymatic reaction in a fluorometer (Photon Technology International, Birmingham, NJ) at 312 nm excitation and 437 nm emission. NAT activity was calculated by reference to a standard curve of Ac-5-ASA fluorescence (0.010–1.0 µM; Pharmacia, Kalamazoo, MI) and normalized to sample protein.

Some experiments varied Ac-CoA (0–0.420 mM) or 5-ASA (0–50 µM). Michaelis-Menten kinetic parameters were calculated by nonlinear regression curve fitting (Prism 4; GraphPad Software, San Diego, CA). In some experiments, reactions additionally contained isoniazid (0.01–10 mM) or p-aminobenzoylglutamate (p-ABG, 0.01–3 mM) (Sigma-Aldrich). Half-maximal inhibitory concentrations (IC50) were calculated by nonlinear regression curve fitting to a one-site competition binding equation (Prism 4). In some experiments testing enzyme stability, homogenates were frozen and thawed multiple times, before analysis.

Measurement of mNAT2 protein. Colon mucosal homogenates were separated using 10% SDS-PAGE (Bio-Rad, Hercules, CA) and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). Membranes were incubated with a blocking buffer (Li-Cor Biosciences, Lincoln, NE) and then probed with rabbit antisera specific for mNAT2 protein (antisera 195; Dr. E. Sim, University of Oxford, Oxford, UK) at 1:10,000 dilution (12) and Alexa Fluor 680 goat anti-rabbit (1:5,000; Invitrogen, Carlsbad, CA). Loading control was β-actin [primary antibody 1:20,000 mouse monoclonal (Cell Signaling Technology, Danvers, MA), secondary antisera 1:10,000 Alexa Fluor 800 goat anti-mouse (Invitrogen)]. Blots were imaged and bands were quantified by an Odyssey Infrared Imager (Li-Cor Biosciences). Background-subtracted mNAT2 integrated intensity was normalized to β-actin to control for protein loading and then normalized vs. an internal control (liver homogenate) present in each gel to allow comparison between membranes.
Cytosolic and membrane protein fractions of colon mucosa homogenate from a distal section were created by a BioVision kit (Mountain View, CA) following the manufacturer’s instructions.

Measurement of mNAT2 mRNA. Muscle-stripped colonic mucosa was collected before tissue homogenization. Total RNA was extracted (TRI Reagent; Molecular Research Center, Cincinnati, OH) and then reverse transcribed (iScript cDNA Synthesis Kit; Bio-Rad). cDNA was amplified by real-time PCR using primers for mnat2 (41) and gapdh using a Fast SYBR Green Master Mix kit (AB Applied Biosystems, Foster City, CA) in a thermal cycler (StepOne Real-Time PCR System, with StepOne Software version 2.1; AB Applied Biosystems). Raw cycle threshold values (Ct values) obtained from mnat target samples were deducted from the Ct value obtained from internal control (gapdh) amplification and calculated relative to a positive control (liver), using the \( \Delta \Delta CT \) method as follows: \( \Delta \Delta CT = (Ct_{target} - Ct_{internal control}) - (Ct_{target} - Ct_{internal control}) \). Final relative values of mRNA abundance were calculated as \( 2^{-\Delta \Delta CT} \).

Statistical analysis. Data are presented as means ± SE for \( n = 6–9 \) independent samples per mice per group. Unpaired two-tailed Student’s t-test was used when SDs were equal between groups. When the SD between groups was significantly different, a nonparametric t-test was performed (Mann-Whitney test). Statistical significance was accepted when \( P < 0.05 \), \( P < 0.01 \), or \( P < 0.001 \). Statistical analyses were always performed comparing control vs. DSS treatment for each colon section. InStat software (version 3.01; GraphPad Software, La Jolla, CA) was used for statistical analysis.

RESULTS

Our initial goal was to establish a simple, sensitive, and specific assay to measure colonic 5-ASA metabolism by NAT enzymes, since existing procedures to measure the Ac-5-ASA metabolite in biological matrices are time consuming and expensive (16, 34, 48–50). We validated an assay based on the intrinsic fluorescence of Ac-5-ASA. As shown in Fig. 1A, 5-ASA and Ac-5-ASA are fluorescent molecules with distinct excitation and emission wavelengths, and 312 nm excitation with 437 nm emission preferentially detects Ac-5-ASA vs. 5-ASA. In the presence of the NAT cofactor Ac-CoA (420 \( \mu \)M) and the substrate 5-ASA (25 \( \mu \)M), the fluorescence immediately (10 s) after adding colon homogenate had an emission spectrum similar to 5-ASA (500 nm; Fig. 1B). The fluorescence intensity increased over time, with a peak emission wavelength similar to Ac-5-ASA (437 nm). To selectively report production of Ac-5-ASA against the background of 5-ASA fluorescence, linear rate of fluorescence increase at 437 nm emission was quantified for the initial 2 min of reaction.

Experiments confirmed that the measured fluorescence increase was dependent on the presence of Ac-CoA, an exogenous source of enzyme (colon homogenate) (Fig. 2A), and was saturable by 5-ASA (Fig. 2B shows a representative experiment). In seven independent experiments using mucosal homogenates from the entire colon, the reaction displayed Michaelis-Menten kinetics with \( K_m \) (5-ASA) = 5.8 ± 0.3 \( \mu \)M, and \( V_{max} \) = 2.6 ± 0.2 \( \mu \)M·min\(^{-1}\)·mg protein\(^{-1}\). Results confirmed that the assay selectively and quantitatively reported enzymatic acetylation of 5-ASA.

Experiments tested the sensitivity and stability of the measurement. As shown in Fig. 3A, the assay reports comparable NAT activity values with input colonic protein from 20 to 150 \( \mu \)g. Results also showed that 5-ASA acetylation rate was not affected when colonic homogenates were kept frozen at \(-80^\circ\)C for up to 4 wk before assay (Fig. 3B). However, enzyme stability was compromised when homogenates were exposed to repeated thaws over the same time interval, with a 14% decrease in activity on the second thaw and a 65% decrease on the fourth thaw (data not shown). All subsequent studies were performed using 75 \( \mu \)g input protein from tissue assayed either fresh or after a single freezing.

**DSS-induced colitis.** We induced colitis in mice by administration of 2.5% DSS in drinking water for 5 days followed by 2 days of regular drinking water before animal death. A low dose of DSS was used to produce modest damage and hopefully reveal differential sensitivity to injury along the colon length. Treated mice had softened stools and anal bleeding. These changes were accompanied by a modest loss of body weight (Fig. 4A) and colonic shortening compared with controls (Fig. 4B). The small effect on these somewhat nonspecific parameters was likely due to the low concentration of DSS that may have more limited toxic effects.

After excision, the colon was divided into three segments (Fig. 5A) so that analyses could be performed in each segment. Figure 5B shows granulocyte infiltration in the mucosa [measured by MPO enzyme activity (40)] increased in each segment after DSS treatment, with the least effect in the proximal colon. MPO activity in the control groups was near the limit of assay detection (2.9 ± 1.4, 3.9 ± 2.7, and 0.1 ± 0.1 mmol H\(_2\)O\(_2\)·min\(^{-1}\)·mg protein\(^{-1}\) in proximal, central, and distal, respectively, \( n = 6 \)). Hematoxylin- and eosin-stained sections

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**Fig. 1.** Fluorescence detection of acetyl-5-aminosalicylic acid (Ac-5-ASA). Representative traces of fluorescence intensity vs. wavelength, recorded in a fluorometer cuvette as described in MATERIALS AND METHODS. A: fluorescence excitation (Ex) and emission (Em) spectra of 100 \( \mu \)M Ac-5-ASA (solid line, peak excitation 312 nm, peak emission 437 nm) or 100 \( \mu \)M 5-aminosalicylic acid (5-ASA, broken line, peak excitation 335 nm, peak emission 500 nm). Excitation spectra were recorded with 437 nm emission, and emission spectra were recorded with 312 nm excitation. Results show relative efficiency for detecting Ac-5-ASA metabolite vs. 5-ASA substrate. B: fluorescence produced over time by in vitro enzymatic reaction in the presence of 25 \( \mu \)M 5-ASA, 0.4 mM acetyl-coenzyme A (Ac-CoA), and whole colon mucosal homogenate. Ac-CoA was added at time 0, and emission spectra were collected at the indicated time.
confirmed segmental heterogeneity of colitis in four mice. Results demonstrate the most severe colitis in central and distal segments.

Effect of DSS colitis on mucosal NAT activity. DSS treatment caused a significant decrease of 5-ASA metabolism (NAT specific activity normalized to protein) in each mucosal segment (48, 62, and 58% decrease in proximal, central, and distal, respectively) compared with the healthy control segment (Fig. 6A). However, we also observed that DSS treatment produced an increase in total mucosal protein content, most prominently in proximal and central segments (data not shown). Therefore, we also quantified total enzyme activity per colon region. In this alternate analysis (Fig. 6B), no change of enzyme capacity was observed in the proximal colon because of DSS treatment, but central and distal colonic segments had decreased enzyme capacity (52 and 51% decrease in central and distal, respectively). Together, these results demonstrate that DSS colitis can decrease 5-ASA metabolism in more distal gut segments and suggest a segmental heterogeneity in this response that qualitatively correlates with colitis severity.

Because 5-ASA is a substrate for all mouse and human NAT isoforms (8, 22, 38) and colonic mucosa expresses at least mNAT1 and mNAT2 (11, 18, 32, 64), we questioned if the basis for altered 5-ASA acetylation during colitis was a change in the contributing mNAT isoform(s). We used substrates shown to be selective for the recombinant mNAT1 (isoniazid) or mNAT2 (p-ABG) (22) and used them as competitive inhibitors of 5-ASA metabolism. In normal colon, both compounds

(Fig. 5C) confirmed segmental heterogeneity of colitis in four mice. Results demonstrate the most severe colitis in central and distal segments.

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decreased 5-ASA metabolism in a concentration-dependent manner (Fig. 7A), suggesting that at least one of the substrates is not perfectly isoform specific or that 5-ASA metabolism in healthy colon is mediated by both enzymes. As shown in Table 1, the observed IC50 (isoniazid) is a poor match with the predicted IC50 for rat NAT1 (4.5-fold different), but the observed IC50 (p-ABG) is close to the predicted IC50 for the human ortholog of mNAT2 (1.5-fold different). The good curve fits to a single inhibitory site model in Fig. 7A, combined with the analysis of inhibitor potency, suggest that mNAT2 mediates 5-ASA metabolism in the colonic mucosa. Next, we tested if 5-ASA metabolism is more or less sensitive to p-ABG after DSS colitis, as would be predicted if disease shifted the relative contributions of mNAT1 vs. mNAT2 to 5-ASA metabolism. In Fig. 7B, the observed IC50 p-ABG concentration was used (436 μM), and the percent inhibition of Ac-5-ASA production was compared between healthy and colitis tissues. DSS caused the proximal colon to be less sensitive to p-ABG, but no changes were caused by DSS in central or distal colon. In a similar protocol, DSS did not cause any difference in isoniazid sensitivity within any segment (data not shown). Results suggest that, at least in central and distal colon, the acetylation of 5-ASA is mediated by mNAT2 in both healthy and inflamed colon.

Effect of DSS colitis on mNAT2 protein and mnat2 mRNA. We quantified mNAT2 by Western blot. The mNAT2 antisera identified two bands in total mucosal homogenate from healthy mice (Fig. 8H), with the predicted mNAT2 band at 31 kDa separating into the cytosolic fraction but not membrane fraction. The pattern of mNAT2 protein expression correlated with enzymatic activity where 5-ASA acetylation was 2.14 ± 0.33 and 5.77 ± 0.75 nmol-min⁻¹·mg⁻¹ in total and cytosolic fractions, respectively, whereas activity was deenriched in the membrane fraction (0.13 ± 0.00 nmol-min⁻¹·mg⁻¹). Based on those data, the lower predominant band was measured as representative for mNAT2. DSS colitis caused a significant reduction in mNAT2 protein in distal colon (54%), a 29% reduction in the central segment that approached significance, and no change in the proximal segment (Fig. 9, A and B).
mediating this metabolic conversion.

5-ASA, and the expression and function of the NAT enzymes tested in mice if acute inflammation affects 5-ASA metabolism (2, 19, 36). We therefore and drug transporters (45, 51, 62, 65), human studies have activity, and functions of many drug-metabolizing enzymes with other minor metabolites (2, 19, 60). Whereas inflammation affects therapeutic outcomes by regulating the expression, conversion to

Crohn’s disease. The pharmacokinetics of the drug include its anti-inflammatory drug to treat mild to moderate UC and has been used for decades (in many formulations) as an

Fig. 6. Effect of DSS colitis on 5-ASA metabolism. Fluorimetric NAT activity assay compares mucosal enzyme activity in control mice (white bars) vs. those exposed to DSS (black bars). Excised colon was divided into proximal, central, and distal segments (see Fig. 5A), and their mucosa homogenate was analyzed separately. Means ± SE for n = 6 (control) or n = 9 (DSS). ***P < 0.01 and ****P < 0.001 comparing DSS treatment vs. corresponding colon segment control. A: NAT metabolism of 5-ASA normalized to the homogenate protein used in the assay. B: NAT metabolism of 5-ASA summmed to estimate the total enzyme activity within a given segment, based on the total amount of protein extracted from each colon segment.

Real-time RT-PCR analysis measured mnat2 mRNA abundance. Results showed no significant effect of DSS colitis on mnat2 mRNA abundance in central and distal segments; however, the proximal segment had a significant 1.8-fold increase of mnat2 mRNA level (Fig. 9C). These results suggest that posttranscriptional regulation of mNAT2 protein may explain any reduced NAT activity in central and distal colon and may also explain the reduced ratio of mNAT2 protein/mRNA in the proximal colon segment.

DISCUSSION

IBD disorders expose patients to the consequences of chronic and episodic inflammation of the gut. The drug 5-ASA has been used for decades (in many formulations) as an anti-inflammatory drug to treat mild to moderate UC and Crohn’s disease. The pharmacokinetics of the drug include its conversion to N-acetyl 5-ASA as the major metabolite, along with other minor metabolites (2, 19, 60). Whereas inflammation affects therapeutic outcomes by regulating the expression, activity, and functions of many drug-metabolizing enzymes and drug transporters (45, 51, 62, 65), human studies have produced only conflicting reports of whether colonic inflammation affects 5-ASA metabolism (2, 19, 36). We therefore tested in mice if acute inflammation affects N-acetylation of 5-ASA, and the expression and function of the NAT enzymes mediating this metabolic conversion.

Table 1. IC50 values for NAT substrates

<table>
<thead>
<tr>
<th>NAT substrate</th>
<th>Observed IC50*</th>
<th>Predicted IC50#</th>
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<tr>
<td>Isoniazid (mNAT1)</td>
<td>5,338 ± 411</td>
<td>1,182</td>
</tr>
<tr>
<td>p-ABG (mNAT2)</td>
<td>436 ± 22</td>
<td>689</td>
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Units are μM. *Values are mean ± SE; values determined from the experiments compiled in Fig. 7. Predicted half-maximal inhibitory concentration (IC50) was calculated assuming competition between 25 μM 5-amino salicylic acid (5-ASA) and the test compounds. This calculation used the observed IC50, the observed Km (5-ASA) = 5.81 μM, and either the Km (isoniazid) for rat N-acetyltransferase (NAT) 1 [223 μM (56)] or the Km [p-aminobenzoylglutamate (p-ABG)] for human NAT1 that is the mouse NAT2 ortholog [130 μM (43)]. Predicted IC50 = [1 + (25/5.8-5)]·Km (competitor).

We developed, validated, and applied a novel fluorimetric rate assay that directly measured production of Ac-5-ASA by NAT enzymes. A limitation of the rate assay format is that it is not suitable to measure steady-state amounts of Ac-5-ASA in tissue extracts. However, the assay reports enzyme activity, is based on the intrinsic fluorescence of Ac-5-ASA, and does not require any processing or chemical conversion of this NAT

Fig. 7. Potency of isoform-specific NAT substrates to block 5-ASA metabolism in healthy and inflamed colonic mucosa. A: Ac-5-ASA production was measured in the presence of the indicated concentration of a selective substrate for mNAT1 (isoniazid, ◦) or mNAT2 [p-aminobenzoylglutamate (p-ABG), ●] as a competitive inhibitor. For each homogenate, results were normalized to the values of Ac-5-ASA production observed in the absence of any inhibitory substance (control). Curves are nonlinear regression fits to a one-site competition binding model, with best-fit values of the inhibitor concentration giving 50% inhibition presented in Table 1. Means ± SE (error bars are smaller than symbols), n = 4 homogenates for each compound. B: Ac-5-ASA production measured in the absence (control) vs. presence of 436 μM p-ABG, and the percent inhibition calculated for homogenates made from healthy colon (white bars) or those exposed to DSS (black bars). Results were measured separately for proximal, central, and distal colon segments as indicated. Means ± SE, n = 6–7 (control) or n = 4–6 (DSS). *P < 0.05 comparing DSS treatment vs. corresponding control.
Peripheral blood mononuclear cells, which downregulate reported evidence for posttranscriptional regulation in human response to the cellular stress from DSS. Butcher et al. also number). This may be due to inflammatory mediators, or a well as potentially decreasing enzymatic efficiency (turnover metabolism by decreasing mNAT2 protein translation or stability, as observed an increase in mNAT2 protein, whereas the observation of posttranscriptional regulation was common among all segments. After DSS, epi-

mNAT2 activity was not due to loss of epithelial cells after DSS sustained in all colon segments, so results suggest that reduced NAT activity was not due to loss of epithelial cells after DSS exposure.

We used a mouse model of acute colitis induced by low DSS and documented the presence of a segmentally heterogeneous colitis so that we could evaluate the segmental heterogeneity of 5-ASA acetylation. In future work, it will be important to ask if similar results are observed in chronic colitis models that more closely resemble human IBD, nonmouse models, and in colitides induced by nonchemical means. DSS treatment reduced Ac-5-ASA production, and we observed that DSS caused a decrease in the ratio of mNAT2 protein/mRNA, suggesting posttranscriptional downregulation of mNAT2 during colitis. This posttranscriptional regulation occurs even during a modest inflammation in the proximal colon, where we observed an increase in mnat2 mRNA but no change in mNAT2 protein. More severe inflammation in the central and distal colon correlated with a significant decrease in total 5-ASA metabolism within a gut segment and a reduction in mNAT2 protein, whereas the observation of posttranscriptional regulation was common among all segments. After DSS, epithelial loss was not noted in the proximal colon by pathological assessment (data not shown), and mnat2 mRNA levels were sustained in all colon segments, so results suggest that reduced NAT activity was not due to loss of epithelial cells after DSS exposure.

Our results suggest that DSS reduces colonic 5-ASA metabol-

ism by decreasing mNAT2 protein translation or stability, as well as potentially decreasing enzymatic efficiency (turnover number). This may be due to inflammatory mediators, or a response to the cellular stress from DSS. Butcher et al. also reported evidence for posttranscriptional regulation in human peripheral blood mononuclear cells, which downregulate hNAT1 activity (the mNAT2 ortholog) after exposure to enzyme substrates (10). Other work shows that activity of recombinant hNAT1 protein is directly inhibited by reaction of biological oxidants with a cysteine in the enzyme active site (6, 15). In contrast, when a mixture of proinflammatory cytokines is added to human cholangiocarcinoma cells for 48 h, hNAT1 activity and mRNA are reduced in parallel (8), an outcome that we did not observe in isolated mouse colonic tissue. We speculate that the observed posttranscriptional regulation of colonic mNAT2 activity may be mediated by increased reactive oxygen species in the inflammatory state.

In conclusion, our study validates a reliable method to directly quantify Ac-5-ASA production that is sensitive enough to measure (at least in theory) 5-ASA metabolism in human colonic biopsies. Results show that, in mouse, an acute colonic

![Fig. 8. Detection of mNAT2 protein in cellular fractions. Representative Western blot membrane visualized on a Li-Cor Odyssey Infrared Imager showing mNAT2 protein (lower predominant red band) and GAPDH (green band) detected on separated proteins of total homogenate (H) and cellular fractions (CF, cytosolic; MF, membrane) from distal colon mucosa of a healthy mouse. NAT2 protein was detected with antisera 195 (kindly provided by Dr. E. Sim, University of Oxford, Oxford, UK). MW, molecular weight marker.](image)

![Fig. 9. Effect of DSS colitis on mNAT2 protein and mRNA levels. A: representative Western blot showing mNAT2 protein (red band) and β-actin (green band) detected on proximal (P), central (C), and distal (D) colon mucosa homogenates from a mouse with DSS colitis. mNAT2 protein was detected with antisera 195, previously validated in mouse tissues (12). L, liver homogenate. B and C: mucosal colonic tissue from healthy animals (control, white bars) was compared with those with DSS colitis (DSS, black bars) with separate analysis of proximal, central, and distal colon segments as indicated. *P < 0.05 and **P < 0.01 comparing DSS treatment vs. corresponding colon segment control. B: Western blot analyses. β-Actin was used as loading control, and liver homogenate was used as a standard for comparison between blots. Means ± SE, n = 6 (control) or n = 9 (DSS). C: real-time RT-PCR measured mnat2 mRNA of colonic mucosa. For quantification, internal control (GAPDH) and positive control (liver) were used, and data were analyzed by the ΔΔCt method. Means ± SE, n = 4 (control) or n = 7 (DSS).](image)
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inflammation impairs the expression and function of mNAT2 enzyme, thereby diminishing the capacity for 5-ASA metabolism. Even a modest inflammation can provoke alterations in the ratio of mNAT2 protein/mRNA. These initial results predict that inflammation will affect the efficacy of 5-ASA therapies against IBD, regardless of whether the effective moiety of therapy is 5-ASA or its metabolite Ac-5ASA. Important areas of future research are to learn whether intracellular drug or metabolite is more active in 5-ASA therapies, and if the same downregulation of 5-ASA metabolism occurs in chronic inflammation. With the use of the new method we introduce, these questions can now be addressed in mice with a confirmed defect in colonic 5-ASA metabolism, or in microscopic samples from human biopsies.

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DISCLOSURES

The authors declare no conflicts (financial, professional or personal) of interest relevant to the conduct of this research.

AUTHOR CONTRIBUTIONS


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