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An Endogenously Anti-Inflammatory Role for Methylation in Mucosal Inflammation Identified through Metabolite Profiling

Douglas J. Kominsky,*† Simon Keely,‡ Melanie Scully,‡ Colm B. Collins,‡ Brittelle E. Bowers,‡ Eric L. Campbell,‡ and Sean P. Colgan‡

Tissues of the mucosa are lined by an epithelium that provides barrier and transport functions. It is now appreciated that inflammatory responses in inflammatory bowel diseases are accompanied by striking shifts in tissue metabolism. In this paper, we examined global metabolic consequences of mucosal inflammation using both in vitro and in vivo models of disease. Initial analysis of the metabolic signature elicited by inflammation in epithelial models and in colonic tissue isolated from murine colitis demonstrated that levels of specific metabolites associated with cellular methylation reactions are significantly altered by model inflammatory systems. Furthermore, expression of enzymes central to all cellular methylation, S-adenosylhomocysteine hydrolase, are increased in response to inflammation. Subsequent studies showed that DNA methylation is substantially increased during inflammation and that epithelial NF-κB activity is significantly inhibited following treatment with a reversible S-adenosylhomocysteine hydrolase inhibitor, DZ2002. Finally, these studies demonstrated that inhibition of cellular methylation in a murine model of colitis results in disease exacerbation while folate supplementation to promote methylation partially ameliorates the severity of murine colitis. Taken together, these results identify a global change in methylation, which during inflammation, translates to an overall protective role in mucosal epithelia. The Journal of Immunology, 2011, 186: 6505–6514.

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shown to be the cell type-specific inhibition of NF-κB activation (19). These studies demonstrate that cellular methylation reactions play an important role in chronic inflammatory disease, such as IBD.

The hypothesis of the current study was that ongoing mucosal inflammatory responses are characterized by specific shifts in metabolic profiles. To define these principles, a nuclear magnetic resonance (NMR)-based metabolomics approach was used to elucidate the metabolic changes associated with murine models of IBD and in cellular models of mucosal inflammation. This analysis revealed that a number of metabolites associated with cellular methylation reactions are significantly impacted by ongoing inflammation. Dovetailing these findings with microarray analysis of epithelial inflammation models, we found that the expression of enzymes central to cellular methylation is significantly altered during mucosal inflammation. We confirmed these findings at the molecular level and extended these studies to a murine model of colitis. These results indicate that methylation reactions play an important role in mucosal inflammation.

Materials and Methods

Cell culture

Human intestinal epithelial cells (T84) and HeLa epithelial cells were grown and maintained in T175 cell culture flasks (Costar, Cambridge, MA) as described previously (22). DZ2002 (Diazyme, San Diego, CA) was prepared as described previously (19). Where indicated, DZ2002 was added at the indicated concentrations by preincubation for 1 h prior to the indicated treatments.

Western blot analysis

Cells were harvested by scraping, pelleted by centrifugation, washed with ice-cold PBS, and lysed by sonication in Tris lysis buffer (150 mM NaCl, 20 mM Tris [pH 5.5], 1 mM EDTA, 1 mM EGTA, and 1% Triton X-100). Similarly, snap-frozen colon tissues samples were sonicated in Tris buffer. Protein concentration was assessed by the bicinchoninic acid assay following the manufacturer’s instructions (Thermo Scientific) to ensure equal protein loading of each preparation. Proteins were separated by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA) for immunoblotting. Abs used for this study were as follows: anti-SAM synthetase (1/1,000; Abcam), anti-SAM synthetase (1/1,000; Bio-Rad Laboratories), anti-IκBα (1/2,000; Cell Signaling Technologies), anti–β-actin (1/10,000; Abcam), anti-β-tubulin (1/10,000; Abcam). Proteins were visualized using the SuperSignal detection substrate (Thermo Scientific).

RNA isolation and transcriptional analysis

RNA was isolated using TRIzol (Invitrogen) and cDNA synthesized as described previously (10). Potential contaminating genomic DNA was digested using Turbo DNA-free (Ambion, Austin, TX). The mRNA profile of intestinal cells was normalized to total protein concentration.

Immunocytochemistry

T84 human epithelial cells were cultured on glass coverslips and fixed in ice-cold methanol, followed by permeabilization in 0.2% Triton X-100. Cells were incubated in 1 M HCl for 1 h at 37°C. Cells were incubated with anti–5-methyl cytidine Ab (Abcam) or IgG control at identical concentrations, followed by application of Alexa Fluor 555 (Invitrogen) secondary Ab. Cells were analyzed using the AxioImager M2 microscope (Carl Zeiss, Oberkochen, Germany). Postanalyses were performed using FlowJo software (Tree Star, Ashland, OR).

Flow cytometry

5-Methyl cytidine staining was quantified using flow cytometry essentially as described by Watson et al. (27). Briefly, cells were trypsinized and fixed in 70% MeOH. Following fixation, cells were incubated in 1 M HCl for 1 h at 37°C. Cells were incubated with anti–5-methyl cytidine Ab (Abcam) or IgG control at identical concentrations, followed by application of Alexa Fluor 555 (Invitrogen) secondary Ab. Cells were analyzed using the FlowJo software (Tree Star, Ashland, OR).

Methylation and Mucosal Inflammation

pNFKB-Luc plasmid transfection and luciferase reporter assay

HeLa cells were passaged into 24-well plates and allowed to attach for 24 h. Transient transfection of Hela cells and assessment of luciferase activity was carried out as described previously (22).

Dextran sodium sulfate colitis model

Dextran sodium sulfate (DSS) colitis was induced with a modification of the technique of Okayasu et al. (25). Colitis was induced on day 0 by the addition of 3% DSS (m.w. = 36,000-50,000; MP Biomedicals, Illkirch, France) solution in drinking water. Control animals received water alone. DZ2002 was delivered i.p. at a concentration of 50 mg/kg/day (19) beginning 1 d prior (day −1) to DSS administration. Folic acid (Sigma-Aldrich) was delivered i.p. at a concentration of 50 mg/kg beginning 1 d prior (day −1) to DSS administration and then every other day for the course of the experiment. BAY 11-7082 (Cayman Chemical) was administered i.p. at a concentration of 5 mg/kg beginning 1 d prior (day −1) to DSS administration and then every other day for the course of the experiment, as described previously (26).

Quantification of IFN-γ in murine colonic tissue

For cytokine analysis, colonic tissue was extracted in Tris lysis buffer by sonication, and protein homogenates were stored at −80°C until use. Tissue concentrations of IFN-γ were measured in colonic protein extracts using a proinflammatory cytokine screen (Meso Scale Discovery). Assays were performed per manufacturer’s instructions and analyzed using a Sector Imager 2400 (Meso Scale Discovery). IFN-γ concentrations were normalized to total protein concentration.

Extraction protocols for metabolic NMR

Collected cell pellets or frozen tumor specimens were homogenized in ice-cold 8% perchloric acid as described previously (28, 29). Briefly, after centrifugation, the supernatants (containing hydrophilic metabolites) were collected, and the pH was adjusted to pH 7 by using KOH. The potassium perchlorate was removed by centrifugation, and the hydrophilic fraction was lyophilized overnight. The cell and tissue pellets (after the first centrifugation), which contained the lipophilic metabolites, were redissolved in water, and the pH was adjusted (7.0). The lipophilic fraction was lyophilized overnight. For the cell experiments only, previously collected media were lyophilized overnight after adjusting pH. The dried hydrophilic cell and tissue extracts were redissolved in 0.5 ml deuterium oxide, transferred into 5-mm NMR tubes, and used for [1H]-, [13C]-, and [15N]-NMR analysis. The medium extracts were redissolved in 1 ml deuterium oxide, transferred to 5-mm NMR tubes, and underwent [1H]- and [13C]-
NMR analysis. The cell and tissue lipid extracts were redissolved in 1.2 ml deuterated chloroform/deuterated methanol mixture (2:1 v/v).

NMR analysis on cell and tissue extracts

All [1H]- and [13C]-NMR spectra were obtained by the Bruker 500 MHz DRX NMR spectrometer using an inverse Bruker 5-mm TXI probe. To assist [1H]-NMR peak assignment and metabolite identification in cell, media and biopsy extracts, two-dimensional-hydrogen/carbon-heteronuclear single quantum correlation NMR techniques were used. All spectra were Fourier transformed, and lactate (Lac3, CH3) was used as an internal chemical reference for both carbon (21 ppm) and proton (1.32 ppm) axes. For metabolite quantification, one-dimensional [1H]-NMR spectra were obtained from each sample, with a standard water presaturation pulse program “zgpr”. A thin sealed glass capillary, containing trimethylsilyl propionate, was placed in each 5-mm tube prior to [1H]-NMR experiments. The total number of acquisitions varied from 40 to 128. Convention [1H] acquisition parameters were power level p1 = 20 dB, power angle p1 = 6.3 ms (90° pulse), power level for water presaturation p9 = 77 dB, water suppression at Q1 = 4.76 ppm, and spectral width SW = 5000 MHz, and the pulse delay of 12.75 s (calculated as 5*T1) was applied between acquisitions. The trimethylsilyl propionate from the reference capillary served as a chemical shift (0 ppm) and proton metabolite concentration reference. The cell and media extracts subsequently underwent [13C]-NMR analysis (with proton decoupling). The total number of scans was 24,000 and 800 for each cell and medium extract, respectively. The concentration of [3-13C] lactate at 21 ppm (calculated from two satellite peaks at the [1H]-NMR spectra) served as an internal carbon concentration reference.

Before [31P]-NMR analysis, 100 mmol/l EDTA was added to the extracts to complex divalent cations (for ATP and ADP quantification). All [31P]-NMR spectra (with proton decoupling) on cell and tissue extracts were obtained by the Bruker 300 MHz Avance NMR spectrometer using a Bruker QNP probe. The total number of scans was 8,000–16,000 per extract. A thin capillary glass containing 2.3 mmol/l methyl-diphosphoric acid served as a chemical shift (18.6 ppm) and phosphor metabolite concentration reference.

Histological scoring

Histological examination was performed on three samples of the distal colon. Samples were fixed in 4% formalin before staining with H&E. All histological quantitation was performed in a blinded fashion, using a previously described scoring system (30). Severity of inflammation: 0, rare inflammatory cells in the lamina propria; 1, increased numbers of inflammatory cells; 2, confluence of inflammatory cells extending into the submucosa; 3, transmural extension of the inflammatory cell infiltrate. Extent of injury: 0, nil mucosal damage; 1, discrete lymphoepithelial lesions; 2, surface mucosal erosion; 3, widespread mucosal ulceration and extension through deeper bowel wall structures. The scores of the two parameters were added and the mean was calculated. Maximum possible score was 3.

Statistics

Unpaired t test and/or one-way ANOVA test were used to determine differences between groups, as indicated. The significance level was set at p < 0.05 for all tests (Prism 4; GraphPad Software, San Diego, CA).

Results

Metabolic profiling of modeled inflammation identifies a “methylation signature”

Previous studies have shown that inflammatory lesions associated with IBD are the site of significant metabolic changes (31–33). These changes in metabolism, however, have not been studied in a systematic manner. In this study, we sought to define the metabolic changes associated with modeled inflammation. Because previous reports strongly implicated intestinal epithelial cells (23, 32), we used T84 cells, a well characterized human intestinal epithelial cell line. Initial experiments involved subjecting T84 human colonic epithelial cells to IFN-γ for 48 h. In addition, cells were cultured in the presence of [1-13C]glucose for the final 4 h of culture to obtain data on the fate of glucose during inflammation. Following the incubation period, cells were immediately harvested and prepared for magnetic resonance spectroscopy (MRS) analysis. The media were also preserved and subjected to metabolite quantification. It has been previously shown that intestinal inflammation results in a state termed “inflammatory hypoxia” (32). In addition, it has been previously shown that treatment of human epithelial cells with proinflammatory cytokines leads to an increase in glycolytic rate (34, 35). The present study corroborates these studies with the finding that treatment of T84 cells with IFN-γ results in an increase in glucose uptake and lactate production as well as intracellular adenosine levels, all of which are indicative of hypoxia (Fig. 1A, Supplemental Fig. 1, Supplemental Table I). Importantly, we also found changes in a number of metabolites that participate in (betaines and cholines) or are products of (creatinine, taurine, and adenosine) cellular methylation reactions (Fig. 1A, Supplemental Fig. 1, Supplemental Table I). These results strongly implicate methylation reactions as a contributing factor to mucosal inflammatory responses. Moving forward, we compared these results with those obtained from microarray analysis (Fig. 1B).

The central enzymes of cellular methylation reactions are induced by IFN-γ

After finding that key metabolites of methylation reactions were changed in our in vitro model of mucosal inflammation, we compared these metabolomic end points with microarray data generated in T84 intestinal epithelial cells following exposure to IFN-γ for either 6 or 18 h. As shown in Fig. 1B, a number of genes strongly implicated in IFN-γ signaling (e.g., CXL11, CXCL10, and IDO1) were highly induced by IFN-γ and thus served as positive controls for this microarray. More relevant for the current study, this microarray also revealed strong induction of several methylation-related gene transcripts, including SAM synthetase (MAT2A), SAH hydrolase (AHCY), O-6-methylguanine-DNA methyltransferase, and guanidinoacetate-N-methyltransferase. MAT2A, the enzyme responsible for SAM production, was among the most highly upregulated genes in the array (Fig. 1B). Given its central role in all methylation reactions (14), we pursued the induction of MAT2A by IFN-γ. To confirm this microarray result, we performed real-time PCR using primers specific for the MAT2A and examined SAM synthetase protein levels by immunoblot analysis. These results indicate that induction of MAT2A expression and SAM synthetase protein are increased in epithelial cells following exposure to IFN-γ (Fig. 1C, for both p < 0.05 by ANOVA). In a similar manner, we also examined the expression of SAH hydrolase. This enzyme plays a key role in methylation reactions by catalyzing SAH, a potent methyltransferase inhibitor, to homocysteine and adenosine (14). These studies revealed that SAH hydrolase expression and protein levels are also upregulated during inflammation (Fig. 1D). Taken together, these results and that of metabolomic analysis provide strong evidence that cellular methylation pathways are important in the inflammatory process.

Increased DNA methylation in a model of epithelial inflammation

Having determined that key enzymes of cellular methylation are upregulated during inflammation, we next examined functional end points of these reactions, namely DNA methylation. For these studies, T84 epithelial cells were exposed to IFN-γ (10 ng/ml) for the indicated times and analyzed levels of 5’-methylcytidine, which is indicative of DNA methylation, using fluorescent microscopy and flow cytometry. As depicted in Fig. 2A, untreated T84 cells exhibit a small amount of nuclear 5’-methylcytidine staining. In stark contrast, as shown in Fig. 2B, nuclear staining for 5’-methylcytidine is substantially increased following exposure to...
IFN-γ for 24 h. This result was corroborated using flow cytometry. As depicted in Fig. 2C, incubation of T84 cells for 24 h with IFN-γ resulted in a 2.23 ± 0.02 increase in 5′-methylcytidine (p < 0.01). An insight into a potential mechanism for the increase in DNA methylation came when the expression of DNMT3b, a DNA methyltransferase responsible for de novo DNA methylation (14),

FIGURE 1. Induction of SAM synthetase and SAH hydrolase during modeled inflammation. T84 human epithelial cells were cultured in the presence of IFN-γ (10 ng/ml) for the indicated times. A displays absolute metabolite concentrations for a subset of the measured metabolites from all treatment groups. B displays a heat-map depiction of gene expression data from microarray analysis showing both control (IFN-γ-inducible) and methylation-related genes. C displays real-time PCR and Western blot analysis for SAM synthetase, and D shows same analysis for SAH hydrolase. PCR data (n = 3; *p < 0.05, ***p < 0.005). Western blot (n = 2).

FIGURE 2. Increase in DNA methylation during inflammation. T84 human epithelial cells were cultured in the presence of IFN-γ (10 ng/ml) for the indicated times. A shows nuclear staining using fluorescent microscopy using an Ab specific for 5′-methylcytidine. B displays nuclear staining for 5′-methylcytidine in T84 cells incubated with IFN-γ for 24 h. C shows flow cytometry data for control or IFN-γ-exposed T84 cells using an anti-5′-methylcytidine Ab or an isotype-matched negative control (n = 3). D depicts real-time PCR data for DNMT3b in control and IFN-γ-exposed T84 cells for 2, 6, 12, and 18 h (n = 3; *p < 0.05, **p < 0.01).
NF-κB activity is modulated in a methylation-dependent manner

NF-κB is one of the master regulators of proinflammatory gene expression (for review, see Ref. 36). NF-κB upregulates a number of genes including proinflammatory cytokines, chemokines, and adhesion molecules and can induce specific sets of genes in response to a particular trigger (37). NF-κB has been shown by EMSA and IHC studies to be highly activated at the site of inflammation in a number of diseases, including IBD (36), as well as activated under conditions of hypoxia (38, 39). Additionally, NF-κB has been found to be protective of the mucosal epithelium in murine disease models (40, 41). Interestingly, NF-κB has been shown to elicit epigenetic modifications of its inducible genes (42). Based on these studies and our preliminary data suggesting a link between methylation and the inflammatory response in epithelial cell models, we examined NF-κB activity using a luciferase reporter assay following inhibition of methylation by DZ2002, a potent reversible SAH hydrolase inhibitor (21). As shown in Fig. 3A, IFN-γ elicited a >8-fold increase in NF-κB activity at the highest concentrations (p < 0.01). Pretreatment with DZ2002 effectively inhibited NF-κB activity in a concentration-dependent manner (Fig. 3A). As IFN-γ does not represent a canonical activator of NF-κB, we also studied the impact of DZ2002 on NF-κB following treatment with TNF-α. As shown in Fig. 3B, although TNF-α is a much more potent NF-κB activator (maximal 58.02 ± 9.66; p < 0.001), inhibition of methylation significantly repressed such induction, likely indicating that this inhibition of NF-κB by DZ2002 is not stimulus dependent.

To confirm that DZ2002 directly impacts NF-κB, we next examined the fate of the NF-κB inhibitory protein, IκBα, in response to DZ2002. As shown in Fig. 3C, IκBα levels are decreased in response to IFN-γ treatment. This response is abrogated with pretreatment with DZ2002 indicating that the inhibition of NF-κB is mediated, at least in part, through modulation of the inhibitory protein IκBα. To confirm these results at a functional level, we examined the expression of TNF-α and IL-8, two known NF-κB target genes. As depicted in Fig. 3D and 3E, expression of both TNF-α and IL-8 is increased in response to IFN-γ treatment. In support of our hypothesis, expression of both of these targets was diminished upon pretreatment with DZ2002 (p < 0.05). These results strongly implicate methylation in NF-κB activation, at least in part, through the regulation of IκBα.

Metabolic profiling of experimental colitis identifies a methylation signature

Previous studies have shown that inflammatory lesions in mucosal inflammation (e.g., IBD) result in substantial metabolic changes and major shifts in metabolite supply and demand (10). In this study, we sought to define the metabolic changes induced during inflammation in an epithelial-driven in vivo model of experimental colitis. For these purposes, we selected the DSS model. C57BL/6 mice were administered DSS or vehicle (water) alone for 5 d, after which mice were euthanized, and colon tissue was immediately removed and frozen. Colon was extracted and prepared for MRS analysis. Fig. 4 displays representative proton spectra for both control (Fig. 4A) and DSS-treated (Fig. 4B) colonic tissue. These experiments revealed alterations in a number of metabolites associated with methylation reactions, including adenosine, betaine,
choline, creatine, glutathione \( (p < 0.05; n = 6) \) (Fig. 4C, Supplemental Table II). In addition to an impact on metabolites involved in methylation, changes in several metabolites are indicative of hypoxia (glucose, lactate, and adenosine), consistent with previous work in experimental colitis models (23, 32, 33, 43). To confirm our in vitro experiments, we examined the parallel expression of SAM synthetase/SAH hydrolase and IFN-\( \gamma \) in colonic tissue extracts from control and DSS-treated mice harvested at 2 and 4 d of treatment. As shown in Fig. 4D, both SAM synthetase and SAH hydrolase expression were increased after 2 and 4 d of DSS administration. Likewise, tissue concentrations of IFN-\( \gamma \) were increased at day 4 of DSS treatment (Fig. 4E).

**Inhibition of methylation exacerbates disease in a mouse model of colitis**

We next examined the impact of methylation-inhibition on DSS colitis outcomes. Given that the SAH hydrolase (AHCY)-null mouse is embryonic lethal (44) and that SAM synthetase is not easily inhibited pharmacologically (15), we reverted to a pharmacological approach using DZ2002. Our in vitro data revealed that modeled inflammation results in an increase in DNA methylation in epithelial models, as evidenced by an increase in 5’-methylcytidine staining detected both by immunocytochemistry and flow cytometry (Fig. 2). On the basis of these findings, we initially examined the extent of DNA methylation in response to DSS in the presence and absence of DZ2002 in vivo. As shown in Fig. 5, treatment with DSS resulted in a significant increase in colonic DNA methylation. Colonic tissue from vehicle-treated animals displayed low levels of basal 5’-methylcytidine staining (Fig. 5, column 1). By contrast, DSS induction resulted in a substantial increase in DNA methylation as indicated by the increase in 5’-methylcytidine and DAPI/5’-methylcytidine costaining (see arrows in merged image, column 2). Treatment of animals with DZ2002 in combination with DSS significantly dampened DNA methylation as indicated by the decrease in both 5’-methylcytidine staining as well as DAPI/5’-methylcytidine colocalization (see arrows in merged image, column 3).

We next demonstrated that DZ2002, administered in combination with administration of DSS, exacerbated disease progression. As shown in Fig. 6A–C, inhibition of methylation in combination with DSS treatment resulted in significant decrease in weight \( (p < 0.05; \text{Fig. 6A}) \), increased colon shortening \( (p < 0.001; \text{Fig. 6B}) \), and an increase in disease activity as indicated by histological score \( (p < 0.001; \text{Fig. 6C}) \). Importantly, DZ2002 treatment alone resulted in no weight loss or colon shortening (Fig. 6A, 6B), indicating that this inhibitor is not toxic at the doses administered. In addition, these indicators of disease severity were confirmed by histology. As shown in Fig. 6D, vehicle-treated mice display normal tissue morphology, including intact epithelium and crypt structure. DSS-treated animals exhibited a deterioration in normal
crypt architecture and epithelial cell depletion (Fig. 6E), and animals treated with DZ2002 and DSS display an almost complete lack of normal tissue architecture and loss of the epithelium (Fig. 6F). These results confirm that inhibition of methylation worsens disease progression in a murine model of colitis and strongly implicate that inflammation-associated methylation functions as an endogenous protective mechanism. Importantly, the finding that NF-κB inhibition exacerbates epithelial-driven colitis is consistent with recent literature (45, 46).

In an attempt to clarify the role of the NF-κB pathway in the context of our findings, we administered the NF-κB inhibitor BAY 11-7082 to mice, alone or in combination with DSS. This inhibitor selectively and irreversibly inhibits NF-κB activation by blocking cytokine-induced phosphorylation of IκB-α without influencing constitutive IκB-α phosphorylation (47). Although animals receiving BAY 11-7082 alone showed no symptomology compared with vehicle controls, mice receiving both BAY 11-7082 and DSS developed significantly more severe disease, to the extent that it

FIGURE 5. DSS treatment increases DNA methylation in vivo. Twelve-week-old C57BL/6 mice were treated as described above. Tissue sections were prepared as previously described (32), followed by labeling with anti-5′-methylcytidine Ab (Abcam) and detection with anti-rabbit Alexa Fluor 555 secondary Ab. Tissues were counterstained with DAPI. Arrows in merged image indicate representative cells displaying staining for both 5′-methylcytidine and DAPI.

FIGURE 6. DSS colitis is enhanced by DZ2002. Twelve- to 18-wk-old C57BL/6 mice were i.p. injected daily with vehicle (0.4% DMSO in PBS) or DZ2002 (50 mg/kg) beginning at day 0. From day 1, mice were administered water (control) or 3% DSS ad libitum for 6 d. A displays percent weight loss, B displays colon length; and C represents histological score (n = 5, data are expressed as mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.005). Histological score determined as described previously (33). D and E, H&E staining of tissue isolated from vehicle (D), DSS (E), and DSS + DZ2002 (F) treated animals.
was necessary that we humanely sacrifice animals prior to completion of the DSS time course (data not shown). These experiments do not provide direct evidence of a role for NF-κB activation in the protection of the mucosa in a DSS model of colitis. However, the results do corroborate our findings and those of others indicating that NF-κB is protective within the intestinal epithelium (40, 41, 48) and suggest that the endogenously protective effect of methylation may be due, at least in part, to epithe
telial NF-κB activation.

**Augmentation with the methyl donor folic acid ameliorates disease in a DSS mouse model of colitis**

Having shown that inhibition of methylation using DZ2002 in combination with administration of DSS worsens colitis in mice, we next determined whether augmentation of methylation would be protective in the DSS model. For these experiments, we administered folic acid in the same manner as the methylation inhibitor (i.e., systemic via i.p. injection) to make the experiments as comparable as possible. As shown in Fig. 7A–C, administration of folic acid in combination with DSS treatment resulted in an increase in weight (p < 0.05; Fig. 7A), significantly decreased colon shortening (p < 0.05; Fig. 7B), and a significant decrease in disease activity as indicated by histological score (p < 0.01; Fig. 7C). Administration of folic acid alone had no effect on weight loss or colon length (Fig. 7A, 7B). Once again, these indicators of disease severity were confirmed by histology. As shown in Fig. 7D, vehicle-treated mice display normal tissue morphology, including intact epithelium and crypt structure. DSS-treated animals exhibited deterioration in normal crypt architecture and epithelial cell depletion (Fig. 7E). Importantly, animals administered folic acid in combination with DSS display improved tissue architecture, diminished loss of the epithelium, and less inflammatory cell migration than DSS alone (Fig. 6F). These results confirm that augmentation of methylation through the administration of folic acid ameliorates disease progression and provides further evidence that inflammation-associated methylation is protective in a murine colitic model. These data are in agreement with clinical studies demonstrating vitamin B12 and folate deficiencies are common in IBD patients (49–51) and suggest folate supplementation in IBD treatment. In addition, a recent study demonstrated that a methyl-deficient diet exacerbates disease in an animal model of colitis (52).

**Discussion**

This study aimed to identify metabolic changes associated with modeled intestinal inflammation, particularly related to the epithelium. As previous work had demonstrated specific shifts in metabolism during inflammation, we reasoned that such changes could be reflected on a more global basis. An amalgamated approach using NMR-based metabolomics and transcriptional arrays identified shifts in methylation-dependent pathways as a major metabolic fingerprint. These studies significantly extend previous work related to inflammation-associated metabolism and identify changes in methylation as a target signature within the epithelium during mucosal inflammation. Evidence is provided that shifts in methylation associated with inflammation serve an endogenously protective role in murine colitis.

Ongoing mucosal inflammatory responses are characterized by significant shifts in tissue metabolism (10). These changes include a shift toward a glycolytic phenotype and careful analysis has shown the development of significant hypoxia, termed inflammatory hypoxia, particularly prominent within the epithelium (53). Coinciding with inflammation-associated hypoxia is the stabilization of hypoxia inducible factor (32, 53), and given the central role of hypoxia inducible factor in most metabolic processes (54, 55), we reasoned that overall metabolism would be changed. In support of this hypothesis, global analysis of metabolism using MRS identified significant changes in a number of cellular

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**FIGURE 7.** DSS colitis is reduced in folic acid-treated animals. Eight- to 10-wk-old C57BL/6 mice were i.p. injected every other day with vehicle (PBS) or folic acid (50 mg/kg) beginning at day 0. From day 1, mice were administered water (control) or 3% DSS ad libitum for 6 d. A displays percent weight loss; B displays colon length; and C represents histological score (n = 4, data are expressed as mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.005). Histological score determined as described previously (33). D and E, H&E staining of tissue isolated from vehicle (D), DSS (E), and DSS + folic acid (F)-treated animals.
metabolites. Many of these alterations are in agreement with results from our laboratory indicating the presence of inflammatory hypoxia. These include increased intracellular adenosine, increased glucose uptake and intracellular glucose, increased intracellular lactate and lactate export, and increased turnover of ATP (Figs. 1, 4, Supplemental Fig. 1, Supplemental Tables I, II). In addition, significant changes were observed in a number of lipid metabolites including monounsaturated fatty acids, triacylglycerol, polyunsaturated fatty acids, phosphatidyl choline, and phosphatidyl ethanolamine (Supplemental Tables I, II), which may be indicative of dynamic alterations in cellular membranes in response to inflammation. Importantly, this analysis revealed a distinct and specific methylation fingerprint. Cellular methylation reactions include modification of DNA, RNA, proteins, and lipids (56, 57). These reactions all require a methyl donor for the modification of the target. The methyl donor for the majority of these reactions is SAM (11). SAM is distributed within all cell and tissue compartments and functions as a methyl donor for a number of different methyltransferases. The donation of methyl groups results in the generation of SAH. Methyltransferase enzymes have a higher affinity for SAH than SAM, and thus, SAH functions primarily as an epithelial irritant to drive permeability-disease models (40, 41). Our findings with DZ20002 significantly inhibit tissue methylation and that such inhibition is associated with exacerbated DSS colitic responses. In addition, augmentation of methylation through the administration of folate ameliorates disease in this colitic model. Whether methylation inhibition would impact other models of intestinal inflammation in the same manner is not currently known.

Taken together, these studies provide a new and compelling role for methylation as an endogenously protective mechanism for mucosal inflammation. In addition, the findings in this study provide a tractable and potentially new therapeutic opportunities for methylation-dependent targets in mucosal diseases such as IBD.

Disclosures

The authors have no financial conflicts of interest.

References


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