Mechanisms of transcriptional modulation of the human anion exchanger SLC26A3 gene expression by IFN-γ

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Diarrhea occurs because of either increased secretion or decreased absorption of water and electrolytes or both. Indeed, the decrease in Cl− absorption was earlier shown in chronic inflamed ileum of rabbit (36), suggesting its contribution to diarrhea. Diarrhea associated with inflammatory bowel diseases (IBD) is characterized by enhanced secretion of high levels of proinflammatory cytokines such as IFN-γ, TNF-α, and IL-1β (4, 20). Previous studies have shown that the proinflammatory cytokine IL-1β decreased DRA mRNA expression in Caco-2 cells (37). Moreover, DRA mRNA expression was found to be significantly reduced in patients with ulcerative colitis (37) and in two animal models of colitis, the IL-10 knockout mouse (22) and the HLA-B27/B2m transgenic rat (15). Our recent studies showed that IFN-γ decreased Cl−/HCO3− exchange activity in human intestinal epithelial cells (33). Moreover, the inhibitory effects of IFN-γ on SLC26A6 gene expression were mediated via interferon regulatory factor 1 (IRF-1) transcription factor (33). Also, we have recently shown that the proinflammatory cytokine IFN-γ decreased both DRA mRNA expression and promoter activity (1). However, the molecular mechanisms involved in the transcriptional modulation of DRA by IFN-γ are not known.

Therefore, the present study was undertaken to elucidate the molecular mechanisms underlying the modulation of intestinal DRA by IFN-γ. Our results demonstrated that IFN-γ decreased DRA promoter activity in Caco-2 cells via the JAK (Janus kinase)/STAT1 pathway. Also, our data showed the involvement of signal transducer and activator of transcription factor 1 (STAT1) in the observed modulation of intestinal DRA gene expression by IFN-γ. These findings provide novel evidence for the involvement of STAT1 in the potential regulation of intestinal chloride absorption in inflammation-associated diarrheal disorders.

MATERIALS AND METHODS

Materials. Human recombinant IFN-γ was obtained from Sigma (St. Louis, MO). γ32P-ATP (3,000 Ci/mmol) was from Amersham (Arlington Heights, IL). JAK inhibitor I was obtained from Calbiochem (San Diego, CA). Polyclonal anti-human STAT1 or normal rabbit IgG antibody and consensus and mutant oligonucleotides for
STAT1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All restriction endonucleases and other modifying enzymes were obtained from New England Biolabs (Beverly, MA), Invitrogen (Gaithersburg, MD), or Promega (Madison, WI). Luciferase assay system was procured from Promega. The β-galactosidase assay kit was obtained from BD Biosciences Clontech (Palo Alto, CA).

Cell culture. Caco-2 cells obtained from the American Type Culture Collection were grown routinely in T-75-cm² plastic flasks in minimum essential medium supplemented with 4.5 g/l glucose, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 10 mM HEPES, 1% essential and nonessential amino acids, and 20% fetal bovine serum, pH 7.4 in 5% CO₂-95% O₂ at 37°C. Caco-2 cells were plated at a density of 1 × 10⁵ cells/cm² on 12-well Transwell collagen-coated inserts (permeable supports) and transfected while still in suspension. For promoter studies, 24 h posttransfection, cells were incubated with IFN-γ (30 ng/ml) for 24 h in serum-reduced medium (1% FBS). For inhibitor studies, transfected cells were pretreated with JAK inhibitor I (30 nM) for 1 h and then coincubated with IFN-γ (30 ng/ml) for another 24 h.

Cell lyses and Western blotting. Caco-2 cells grown to confluence in 12-well Transwell collagen-coated inserts (Corning Costar; Lowell, MA) were serum starved overnight and treated with IFN-γ (30 ng/ml) for 24 h. Cells were washed with ice-cold PBS three times and lysed in 20 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% Triton-X 100, 1 mM EDTA, 1 mM EGTA and 1× complete protease inhibitor cocktail (Roche, Indianapolis, IN). The cells were homogenized by passing 10 times through a 26-gauge needle. The lysate was centrifuged at 5,000 g for 5 min at 4°C, and protein concentration was determined by the method of Bradford (5). To detect DRA expression in untreated and IFN-γ-treated confluent Caco-2 cells, equal amounts (~75 μg/sample) of whole cell lysates were solubilized in SDS-gel loading buffer and boiled for 5 min. Proteins were separated on 10% SDS-PAGE gels and probed with human anti-DRA as previously described (12). Bands were visualized with enhanced chemiluminescence detection reagents.

Reporter plasmid construction. Plasmids used for functional analysis of the DRA promoter activity were generated by using pGL2 basic vector (Promega) that contains a promoterless luciferase reporter gene. Utilizing the DRA promoter construct (p-1183/+114; 1.3-kb fragment) as template, we generated four 5'-deletion constructs of p-790/+114, p-398/+114, p-179/+114, and p-44/+114 by the PCR amplification method. Four different forward primers contained an internal site for KpnI restriction enzyme and their sequences are primer 1, 5'-GGGTTACCAAGAAGAGATTTTAGCCCGCATGAC-3'; primer 2, 5'-GGGTTACCGACCCCATGACAAGTCTCATGAC-3'; primer 3, 5'-GGGTTACCTGGAGGAACATCTGATGTC-3'; and primer 4, 5'-GGGTTACCGTTCAATGACGCGTATATAGAC-3'. The sequence of the reverse primer contained site for BglII enzyme was 5'-GGGATCCTAATACGACGATCATGTATGGAAGA-3'.

The amplifications were performed by using proofreading Elongase enzyme mix (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PCR products were then digested with KpnI and BglII enzymes and cloned into luciferase reporter gene vector, pGL-2 basic (Promega). The fidelity of the constructs was then confirmed by sequencing, and plasmids were prepared for transfection by use of a kit from Qiagen (Valencia, CA).

Site-directed mutagenesis. Site-directed mutations was carried out in the critical potential binding site [γ-activated sequence (GAS)] for transcription factor STAT1 (~933 to ~925 bp) by using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions and confirmed by sequencing. The bold letters indicate the mutations. The GAS mutant oligonucleotide used was 5'-TCTTTGCATGGACCGATAGTGGTGGTCGGAAGTAAGGCTAGCTAGGAA-3'.

Transient transfection and luciferase assays. For transfection studies, Caco-2 cells were transfected by using an Amaza Nucleofector System according to the manufacturer’s instructions. Briefly, ~1 × 10⁶ cells were harvested and then electroplated in 100 μl of solution T (supplied by Amaza) with one of the DRA promoter-luciferase constructs (30 μg) and 2.0 μg of pCMVβ (β-galactosidase mammalian expression vector, Clontech, Mountain View, CA). Cells were transfected to full media and plated on 12-well Transwell collagen-coated inserts (permeable supports). At 48 h posttransfection, cells were lysed in reporter lysis buffer (Promega) and the activities of both firefly luciferase and β-galactosidase were measured by luminometer according to the manufacturer’s instructions with kits from Promega and Clontech, respectively. The promoter activity was expressed as a ratio of luciferase to β-galactosidase activity (relative luciferase activity) in each sample.

Nuclear extracts and EMSA. Nuclear extracts were prepared from control or IFN-γ (30 ng/ml)-treated Caco-2 cells as previously described (33). The sequence of the top strand of the probe containing GAS motif (shown in bold) 5'-TCTTTTAGGGTAAAGAGGGTTAGGA-3' spanned from nucleotides ~939 to ~928. GAS double-stranded oligonucleotide was end labeled with T4-polynucleotide kinase and γ³²P-ATP (Amersham, Arlington Heights, IL). DNA/protein binding reactions were performed as previously described (33).

ChIP. Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP-IT Express kit from Active Motif (Carlsbad, CA) as previously described (33). Briefly, untreated or IFN-γ-treated confluent Caco-2 cells (grown in 6-well Transwell collagen-coated inserts) were cross-linked with 1% formaldehyde for 10 min at room temperature (21–23°C). Chromatin fragments were isolated from nuclei by enzymatic shearing (200–800 bp), and protein concentration of the sheared chromatin lysate was determined by the method of Bradford (5). The sheared chromatin protein (untreated and IFN-γ treated, 500 μg each) was used for immunoprecipitation with 3 μg of STAT1 or normal rabbit IgG antibody and 25 μl of protein G magnetic beads. A no-antibody control was also used. Immunoprecipitation was carried out for 4 h on a rotator at 4°C. Immunoprecipitated DNA samples were purified (Qiagen) and used for real-time PCR amplification. The resulting purified DNA fragments were subjected to real-time PCR for the quantification of association of STAT1
IFN-γ inhibits DRA expression. Recently, we have shown that IFN-γ decreased Cl⁻/OH⁻ exchange activity in Caco-2 cells (33). IFN-γ decreased DRA mRNA expression and promoter activity in Caco-2 cells (1). We examined effects of IFN-γ on DRA protein levels in Caco-2 cells. Western blot analysis showed that IFN-γ (30 ng/ml, 24 h) significantly decreased DRA protein levels in Caco-2 cells (Fig. 1A, ~40% decrease compared with control, Fig. 1B). Thus decrease in Cl⁻/OH⁻ exchange activity in Caco-2 cells by IFN-γ was found to be consistent with decreased DRA protein expression.

Identification of the IFN-γ-responsive region in DRA promoter. In an effort to determine which region of DRA promoter is responsible for IFN-γ-mediated inhibition of DRA promoter activity, a series of 5'-truncated DRA-reporter constructs were generated in pGL2 basic vector containing progressive deletions from the 5'-end of the full-length DRA promoter construct, p-1183/+114. Figure 2 depicts the promoter activity of the full-length (p-1183/+114) and other 5'-deletion constructs in response to IFN-γ (30 ng/ml, 24 h). The full-length promoter construct, p-1183/+114, exhibited ~50% inhibition in promoter activity in response to IFN-γ treatment compared with untreated control. However, deletion from -1183 to -790 (p-790/+114) abolished the inhibitory effects of IFN-γ. Similar results were obtained with the other deletion constructs, p-398/+114 and p-179/+114. However, p-44/+114 did not show any basal DRA promoter activity compared with the pGL2 basic empty vector (data not shown). These results suggested that the IFN-γ-responsive element(s) is located between -1183 to -790 region.

IFN-γ effects are JAK and STAT1 dependent. IFN-γ is known to mediate its effects through the JAK/STAT1 (25). We next investigated the role of JAK and STAT1 in mediating the with the DRA promoter in untreated and IFN-γ-treated samples by using primers (GAS) flanking the potential GAS binding sequence (forward: 5'-CTATGGGCAATCAAGCA-3'; reverse: 5'-TGTTCT-GAATTTGCTTCCTCGTGC-3') and primers (non-GAS) that do not contain the GAS site and are ~0.4 kb away from this site (forward: 5'-GATTCGACAGATGACATTGTCGTGCTTTT-3'; reverse: 5'-GATGGACAGTTTGACGTGGCTTTT-3'). The quantification of STAT1 protein binding to the promoter region was expressed as ratio of 2^ΔΔCt_{untreated/ΔΔCt_{IFN-γ}} as previously described (26, 33). ΔCt was obtained by subtracting the threshold cycles (Ct) of STAT1 DNA immunoprecipitates amplified from untreated and IFN-γ-treated samples by using non-GAS primers (away from GAS site) from the same corresponding samples by using GAS primers flanking the GAS site (~933/~925 bp). The relative binding association was expressed as fold increase in arbitrary unit with control set as "1".

Statistical analysis. Results are expressed as means ± SE. Student’s t-test was used in statistical analysis. P < 0.05 or less was considered statistically significant.

RESULTS

IFN-γ inhibits DRA expression. Recently, we have shown that IFN-γ decreased Cl⁻/OH⁻ exchange activity in Caco-2 cells (33). IFN-γ decreased DRA mRNA expression and promoter activity in Caco-2 cells (1). We examined effects of IFN-γ on DRA protein levels in Caco-2 cells. Western blot analysis showed that IFN-γ (30 ng/ml, 24 h) significantly decreased DRA protein levels in Caco-2 cells (Fig. 1A, ~40% decrease compared with control, Fig. 1B). Thus decrease in Cl⁻/OH⁻ exchange activity in Caco-2 cells by IFN-γ was found to be consistent with decreased DRA protein expression.

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effects of IFN-γ on DRA promoter activity. Transiently transfected Caco-2 cells were treated with the specific JAK1 and 2 inhibitor, JAK inhibitor I (30 nM), or specific STAT1 inhibitor, EGCG (50 μM), for 1 h prior to the addition of IFN-γ (30 ng/ml), followed by coincubation at 24 h. EGCG is known to inhibit STAT1 phosphorylation in Caco-2 cells (28). Both JAK inhibitor I and EGCG significantly blocked the IFN-γ-mediated effects (Fig. 3, A and B), indicating that JAK/STAT1 pathway is involved in IFN-γ effects. JAK phosphorylates STAT1 to activate it. Activated STAT1 then moves from the cytoplasm to the nucleus, where it binds to the GAS of the promoter to affect gene transcription (7, 10). Interestingly, sequence analysis of the IFN-γ-responsive region (p-1183/−790) revealed a potential GAS element for transcription factor, STAT1.

Fig. 4. IFN-γ-induced STAT1 interacts with the potential γ-activated site (GAS) motif. The DRA promoter region (p-1183/+114) depicts the various transcription factors including the GAS element located within the −933 to −925 region (A). EMSA was performed by using a double-stranded oligonucleotide (−939 to −928 bp) as end-labeled probe and nuclear extracts from untreated (control) or IFN-γ (30 ng/ml)-treated Caco-2 cells. A total of 10 μg of nuclear proteins were combined with 50,000 cpm probe per reaction and after 30 min incubation at room temperature resolved on a 4% nondenaturing polyacrylamide gel and visualized by autoradiography (B). Lane 1 depicts only probe. DNA-protein binding in control (lane 2) was significantly increased (lane 3) in response to IFN-γ. Competition experiments were performed in the presence of unlabeled cold GAS oligo (lane 4), consensus STAT1 oligo (lane 7), cold mutant GAS oligo (lane 5), cold STAT1 mutant oligo (lane 6), and unrelated NF-κB oligo (lane 8). Anti-STAT1 antibody (2 μg, lane 9) blocked the formation of the DNA-protein complex. The + and − (shown on top) signs indicate the presence or absence of reaction components in the reaction mixture. Gels are shown as a representative of 3 separate experiments with similar results.
IFN-γ induces STAT1 transcription factor that binds to potential GAS element in the DRA promoter. We next examined the ability of the potential GAS cis element to bind STAT1, and we performed EMSA using the GAS cis element as an end-labeled probe. As shown in Fig. 4, the incubation of the 32P-labeled GAS cis element with the nuclear extracts from untreated (control) or IFN-γ-treated cells resulted in two bands representing binding of the probe to STAT1. The binding of labeled potential GAS site to Caco-2 proteins (DNA-protein complexes) was significantly increased in the presence of IFN-γ (lane 3) compared with control (lane 2). The binding specificity of the complexes was examined by competition experiments in which excess of unlabeled cold GAS or mutant oligo or consensus or mutant STAT1 oligo was used. The DNA-protein complex was eliminated in the presence of cold unlabeled probe (lane 4) or consensus STAT1 oligo (lane 7), but not in the presence of mutant GAS oligo (lane 5) or mutant STAT1 oligo (lane 6) and an unrelated NF-κB oligo (lane 8). To confirm the identity of the protein in these complexes, we added the specific STAT1 antibody. Although no supershift band was observed, addition of the anti-STAT1 antibody in high concentration blocked the formation of both the DNA-protein complexes (lane 9). Addition of an antibody in EMSAs may result in blocking the formation of the DNA-protein complex if the antibody binds to a site on the transcription factor that is essential for DNA binding (24). Thus our results (lane 9) suggested a blocking of the complexes by the antibody, indicating that STAT1 binds to the potential GAS element of DRA promoter. These results further indicate the role of STAT1 in IFN-γ-mediated inhibition of DRA promoter activity.

IFN-γ induces association of STAT1 with the SLC26A3 promoter in vivo. EMSA showed that STAT1 binds to the potential GAS element of DRA promoter region. The interactions of STAT1 transcription factor with the DRA promoter in vivo were further confirmed by ChIP assays. Untreated and IFN-γ-treated Caco-2 cells were cross-linked by use of formaldehyde, and sheared chromatin was isolated and subjected to immunoprecipitation using anti-STAT1 or normal rabbit IgG antibody. Immunoprecipitated DNA was purified and subjected to real-time PCR using GAS primers flanking the GAS element and non-GAS primers (≈0.4 kb away from the GAS element). Figure 5A shows PCR products of expected size amplified by primers flanking the GAS element by using normal rabbit IgG or STAT1 immunoprecipitated DNA as template. Real-time PCR results showed that the value of ΔC_{T(EGR-STAT1)}, i.e., differences in the C_T of amplification between IgG and STAT1 DNA-immunoprecipitates with the non-GAS primers in both untreated and IFN-γ-treated samples, was “0,” indicating that there is no enrichment of the DNA fragment away from the STAT1 binding region. However, with the GAS primers, ΔC_{T(EGR-STAT1)} value was “1” in untreated and “2” in IFN-γ-treated samples, indicating a two- and fourfold enrichment of the DNA fragment flanking the STAT1 binding region in untreated and IFN-γ-treated samples, respectively. Therefore, our results showed that IFN-γ increased the association of STAT1 with DRA promoter region (containing GAS element) by approximately twofold compared with untreated control (Fig. 5B). These findings further confirm the role of STAT1 in the inhibitory effects of IFN-γ effects on DRA promoter activity.

Potential GAS element is essential for IFN-γ-mediated inhibition of DRA promoter activity. To confirm the role of STAT1 in the inhibition of DRA promoter activity by IFN-γ, we generated mutations in the potential GAS element. As shown in Fig. 6, mutations in the GAS element (shown in shaded box) significantly abrogated the inhibitory effects of IFN-γ on DRA promoter activity. These results further demonstrated that the GAS cis element may be involved in the modulation of DRA promoter in response to IFN-γ.

DISCUSSION
In the human intestine, electroneutral NaCl absorption is mediated by the dual operation of two apical membrane Na+/H+ (NHE3/NHE2) and Cl−/OH− (HCO3−) exchangers (DRA/PAT1). Recent studies have demonstrated repression of...
this region also showed potential binding sites for other transcription factors such as AP1, NF-κB, and C/EBPβ in addition to the GAS element (STAT1). Therefore, we initially performed gel shift assays to assess the ability of these potential sites to bind Caco-2 nuclear proteins in the presence and absence of IFN-γ. Only a minimal increase in NF-κB/DNA binding activity was observed in response to IFN-γ, compared with the potential GAS element, and IFN-γ did not show any change in AP1/DNA binding activity (data not shown). Furthermore, the involvement of C/EBPβ in IFN-γ-mediated effects was ruled out because previous studies have shown that C/EBPβ-induced gene expression by IFN-γ was independent of JAK1 but required STAT1 (19). In contrast, our studies showed that the inhibitory effects of IFN-γ on SLC26A3 promoter were mediated through both the JAK and STAT1 pathway. On the basis of these results, we concluded that, compared with the other potential binding sites, GAS element may be important for IFN-γ-mediated downregulation of SLC26A3 promoter activity in Caco-2 cells. Interestingly, mutation in the potential GAS cis element attenuated the inhibitory effects of IFN-γ on promoter activity (Fig. 6) in parallel with blocking the STAT1 binding to GAS probe by the mutant STAT1 oligo (Fig. 4, lane 6). Additionally, EMSAs showed increased protein binding to the oligonucleotide spanning the potential GAS element (−933 to −925 bp) under IFN-γ-stimulated conditions (Fig. 4, lane 3). The DNA-protein complex was blocked by STAT1 antibody, suggesting that the identified protein is STAT1 (Fig. 4, lane 9). These findings were substantiated in vivo by performing ChIP assays coupled with real-time PCR analysis in control and IFN-γ-treated

NHE3 and PAT1 promoter activity in human intestinal epithelial cells by the proinflammatory cytokine IFN-γ by distinct mechanisms (3, 33). Also, recently we have shown that IFN-γ inhibited DRA expression and promoter activity in Caco-2 cells (1). However, the mechanisms underlying the inhibition of DRA gene expression by IFN-γ in the intestine were not known. In the present study, we have elucidated the signaling pathways, cis element(s), and transcription factor(s) involved in the inhibition of DRA expression in response to IFN-γ. IFN-γ exerts its effects via the induction of signal transduction pathway involving JAK1 and 2 and STAT1. Both JAK1 and JAK2 bind specifically to the intracellular domains of IFN-γ receptor signaling chains, resulting in the phosphorylation of JAKs and intracellular tyrosine residues on IFN-γ receptor to form STAT docking sites (27). Tyrosine phosphorylation of STAT1 leads to STAT1 homodimerization. Activated STAT1 dimers move from the cytoplasm to nucleus, where they bind to 8- to 10-bp inverted repeat DNA element with a consensus sequence of 5′-TT(N4–6)AA-3′ known as the GAS element (7).

By using the specific JAK1 and 2 inhibitor JAK inhibitor I and the STAT1 inhibitor EGCG, the inhibitory effects of IFN-γ on DRA promoter activity were completely abrogated, indicating the involvement of JAK/STAT1 pathway. In fact, previous studies have shown that EGCG attenuated the activation of phospho-STAT1 and inhibition of NHE activity in Caco-2 cells in response to IFN-γ (28). Progressive deletions from the 5′-flanking region of DRA promoter showed that the IFN-γ-responsive region is located between −1183 to −790 bp region given that deletions from −1183 to −790 bp significantly abrogated the inhibitory effects of IFN-γ on DRA promoter activity. Sequence analysis of the IFN-γ responsive region identified one potential GAS cis element flanking the region of −933 to −925 bp. However,

![Fig. 6. Potential GAS element is the IFN-γ-response motif. Caco-2 cells were transiently transfected with DRA promoter construct (−1183/+114) and a construct with a mutated GAS element (GAS mutant; mutated sites are shown as shaded box) along with pCMVβ-galactosidase vector. At 24 h posttransfection, cells were then treated with 30 ng/ml of IFN-γ for 24 h in media containing 1% FBS. Cells were then harvested 48 h posttransfection and the promoter activity was measured by luciferase assay. Values were normalized to β-galactosidase activity to correct for transfection efficiency. Results represent means ± SE of 4 separate experiments performed in triplicate and are expressed as % of control comparing transfected cells treated with IFN-γ with untreated cells (control). *P < 0.05 compared with control.](image1)

![Fig. 7. Proposed model of the potential mechanisms of IFN-γ-mediated inhibition of DRA gene expression in human intestinal epithelial cells.](image2)
Caco-2 cells. We showed that the association of STAT1 with endogenous DRA promoter, which contains the GAS element (Fig. 5B), was significantly increased in the presence of IFN-γ compared with control. Our studies thus provide evidence for the role of STAT1 in the regulation of DRA promoter activity. Our results are in accordance with previous studies of Schreiber et al. (34) showing that increased expression and activation of STAT1 in the colonic mucosa of patients with active ulcerative colitis may play an important role in the pathophysiology of colonic inflammation (32). These published results further lend support to the important role in the pathophysiology of diarrhea associated with IBD.

We have recently shown that IFN-γ decreased Cl-/OH- exchange activity in Caco-2 cells via a JAK-dependent pathway (33). Also, IFN-γ significantly inhibited PAT1 and DRA expression and promoter activity in Caco-2 cells (1, 33). Hence, IFN-γ-mediated decrease in Cl-/OH- exchange activity in these cells could be a function of both PAT1 and DRA expression. Taken together, these observations indicate that the GAS element on DRA promoter appears to play an important role in the regulation of DRA gene expression by STAT1. In contrast to present studies, our studies of modulation of PAT1 and NHE3 promoter activity by IFN-γ showed involvement of IRF-1 (33) and Sp1 and Sp3 (3) transcription factors, respectively, rather than STAT1.

A schematic representation of the involvement of STAT1 in the regulation of intestinal DRA gene expression by IFN-γ is shown in Fig. 7. This inhibition occurs via a putative GAS cis element in the DRA promoter. We speculate that in inflammatory conditions the repression of both DRA and PAT1 promoter activity along with NHE3 promoter by IFN-γ may lead to decreased NaCl absorption in the ileum and colon. This overall decrease in NaCl absorption in the intestine may lead to the pathophysiology of diarrhea associated with IBD.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


