GPR109A Is a G-protein–Coupled Receptor for the Bacterial Fermentation Product Butyrate and Functions as a Tumor Suppressor in Colon

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Abstract

Short-chain fatty acids, generated in colon by bacterial fermentation of dietary fiber, protect against colorectal cancer and inflammatory bowel disease. Among these bacterial metabolites, butyrate is biologically most relevant. GPR109A is a G-protein–coupled receptor for nicotinate but recognizes butyrate with low affinity. Millimolar concentrations of butyrate are needed to activate the receptor. Although concentrations of butyrate in colonic lumen are sufficient to activate the receptor maximally, there have been no reports on the expression/function of GPR109A in this tissue. Here we show that GPR109A is expressed in the lumen-facing apical membrane of colonic and intestinal epithelial cells and that the receptor recognizes butyrate as a ligand. The expression of *GPR109A* is silenced in colon cancer in humans, in a mouse model of intestinal/colon cancer, and in colon cancer cell lines. The tumor-associated silencing of *GPR109A* involves DNA methylation directly or indirectly. Reexpression of GPR109A in colon cancer cells induces apoptosis, but only in the presence of its ligands butyrate and nicotinate. Butyrate is an inhibitor of histone deacetylases, but apoptosis induced by activation of GPR109A with its ligands in colon cancer cells does not involve inhibition of histone deacetylation. The primary changes in this apoptotic process include down-regulation of Bcl-2, Bcl-xL, and cyclin D1 and up-regulation of death receptor pathway. In addition, GPR109A/butyrate suppresses nuclear factor-κB activation in normal and cancer colon cell lines as well as in normal mouse colon. These studies show that GPR109A mediates the tumor-suppressive effects of the bacterial fermentation product butyrate in colon. [Cancer Res 2009;69(7):2826–32]

Introduction

The mammalian colon is the home for billions of bacteria, and these bacteria markedly influence the biology of the host, including energy balance, gene expression, immune function, and disease processes (1–3). Dietary fiber is fermented by bacteria in the colonic lumen to generate short-chain fatty acids, which are responsible for the beneficial effects of gut bacteria on intestinal/colonic health (4, 5). The presence of short-chain fatty acids in the colonic lumen is linked to decreased incidence of colorectal cancer and inflammatory bowel disease (6–9), but little is known on the molecular mechanisms involved in the maintenance of colonic health by these bacterial products. One of these short-chain fatty acids is butyrate, which functions as a tumor suppressor by inhibiting histone deacetylases (HDAC; refs. 10, 11). Recently, we and others have identified a Na^+-coupled transporter for short-chain fatty acids (12, 13). This transporter, known as SLC5A8, recognizes short-chain fatty acids and other monocarboxylates as substrates (12–17). SLC5A8 was originally identified as a tumor suppressor in colon (18) and its expression is silenced in colon cancer (18–20). The discovery that SLC5A8 is an active transporter for butyrate explains, at least partly, its tumor-suppressive function in colon (21–23). SLC5A8-mediated entry of butyrate from lumen into colonic epithelial cells leads to HDAC inhibition and tumor suppression. The expression of SLC5A8 in the lumen-facing apical membrane of colonocytes supports this mode of action (17, 19, 24).

Studies on the tumor-suppressive function of butyrate have focused thus far mostly on its intracellular action as an HDAC inhibitor. Here we show that butyrate also elicits effects in colon cells extracellularly by serving as a ligand for GPR109A. GPR109A is a receptor for nicotinate (niacin) and mediates the lipid-lowering actions of the vitamin (25–27). Recently, Taggart and colleagues (28) showed that the ketone body β-D-hydroxybutyrate is a ligand for the receptor at physiologic concentrations. Butyrate was also able to activate the receptor with an EC_{50} (i.e., concentration necessary for half-maximal activation of the receptor) of ~1.6 mmol/L. Although the levels of butyrate in circulation are too low (~5 μmol/L) to activate the receptor, butyrate is present at high levels (~20 mmol/L) in colonic lumen (29). If GPR109A is expressed in the lumen-facing apical membrane of colonocytes, it might suggest that the ability of butyrate to prevent cancer and inflammation in the colon may also be mediated extracellularly via the receptor without entering into cells. This rationale formed the basis of the current investigation.

Materials and Methods

Immunohistochemistry. Polyclonal antibodies against GPR109A and GPR109B were generated in rabbits. The antigenic peptides used were RKKTLGEPDNNRSTSVC and CHQEPASLEKQLG, respectively. There is only a single gene coding for nicotinate receptor (GPR109A) in mouse (30); therefore, mouse intestinal and colonic tissues were examined only for GPR109A. Human colonic biopsies were obtained during colonoscopy after obtaining patients' informed consent and approval from the institutional review board. Because there are two genes in humans coding for GPR109

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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(GPR109A and GPR109B; ref. 30), human tissues were examined for both isoforms.

Nicotinate binding. Membranes prepared from CCD841 cells (a human colonic cell line) were used for [^{3}H]nicotinate binding assays with a rapid filtration method (31). The interaction of butyrate with the receptor was evaluated by its ability to compete with nicotinate for binding.

Functional analysis of GPR109A. Activation of GPR109A with its ligands was examined using the activity of G-protein–coupled inwardly rectifying potassium (GIRK) channels as the readout. HEK 293 cells were transfected with a human GPR109A expression vector and used for experiments 48 h later. Activation of GIRK channels by adenosine receptor A1R was used as a positive control. A cytosolic GFP expression vector (Invitrogen) was cotransfected with A1R or GPR109A expression vectors to identify transfected cells. When indicated, cells were pretreated overnight with pertussis toxin (100 ng/mL). GIRK channel activity was monitored as described previously (32).

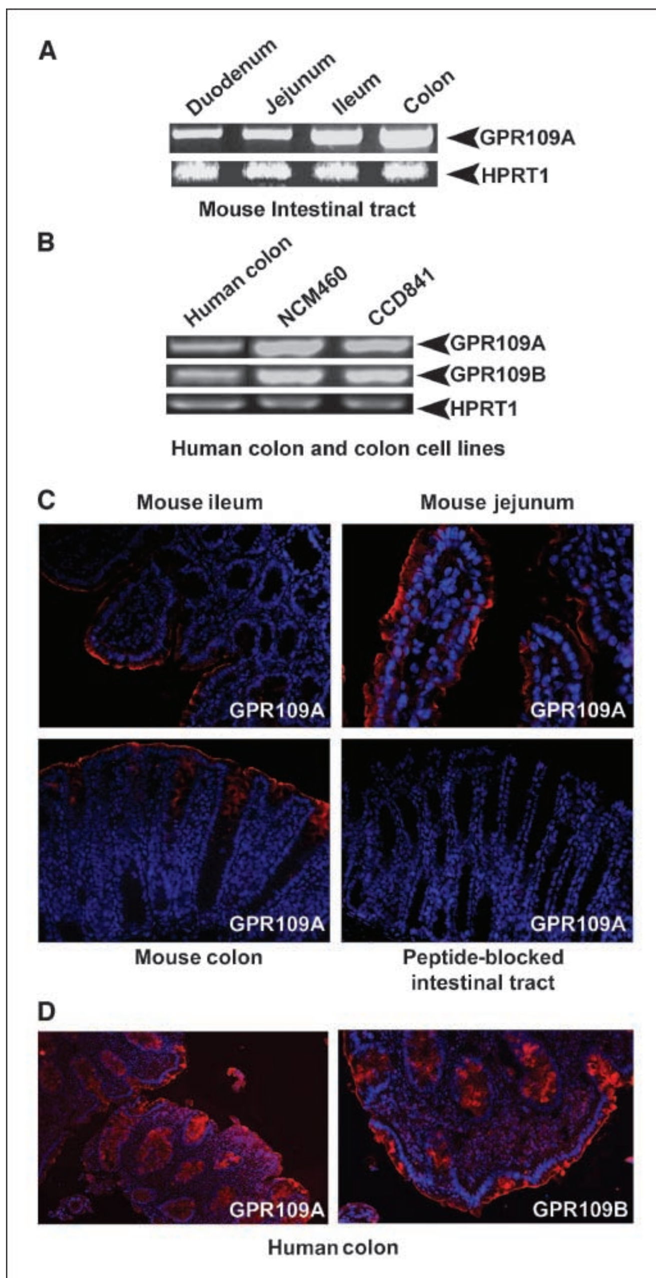

Figure 1. Expression of GPR109A in colon. *A,* expression of GPR109A mRNA in mouse intestinal tract. *B,* expression of GPR109A and GPR109B mRNA in human colon and in human colon cell lines. *C,* immunolocalization of GPR109A protein in mouse intestinal tract (*red,* GPR109A; *blue,* nuclei). *D,* immunolocalization of GPR109A and GPR109B in human colonic biopsies (*red,* GPR109A and GPR109B; *blue,* nuclei).

Tissue collection. Paired normal colon and colon tumor specimens were collected from 18 adult patients with colorectal cancer, with patients' informed consent and approval from the institutional review board (33). Wild-type and $Apc^{Min/+}$ mice were euthanized by CO_2, and tissues from the intestinal tract were collected for RNA preparation.

Reverse transcription-PCR. The PCR primers for specific genes were designed based on the nucleotide sequences available in GenBank. Reverse transcription-PCR (RT-PCR) was repeated twice with each RNA sample. Hypoxanthine phosphoribosyltransferase (HPRT) was used as the internal standard. The intensities of the bands corresponding to specific gene transcripts were quantified by densitometry scanning and normalized to the intensities of corresponding HPRT bands.

Ectopic expression of human GPR109A. Cells (CCD841, a human normal colon cell line, and KM12L4, a human colon cancer cell line) were transfected with pcDNA or human GPR109A cDNA. pEGFP-N1 was used for cotransfection to determine transfection efficiency. After 24 h, cells were treated with or without butyrate (1 mmol/L) or nicotinate (1 mmol/L) for 24 h. Preparation of RNA and protein lysates was done as described previously (20). For fluorescence-activated cell sorting (FACS) analysis, cells were fixed in 50% ethanol; treated with 0.1% sodium citrate, 1 mg/mL RNase, and 50 μg/mL propidium iodide; and subjected to FACS.

Western blot analysis. Fifty micrograms of protein were fractionated by SDS-PAGE, and the fractionated proteins were transferred onto a nitrocellulose membrane (Schleicher & Schull). Membranes were blocked with bovine serum albumin and then exposed to respective primary antibodies at 4°C overnight, followed by treatment with appropriate secondary antibodies. Proteins were visualized by ECL SuperSignal Western System (GE Healthcare).

HDAC activity. A commercially available kit (BioVision) was used to determine HDAC activity in a cell-free system (34). The acetylation status of histone H3, histone H4, histone H4-Lys^{12}, and histone H4-Lys^{16} was assessed by Western blot with specific antibodies as described previously (34). For normalization, the protein levels of histone H3 and histone H4 were determined with specific antibodies. The antibodies were obtained from the following sources: histone H3, acetylated histone H3, histone H4, and acetylated H4 (Upstate Biotechnology, Inc.); acetylated H4-Lys^{12} (Santa Cruz Biotechnology, Inc.); and acetylated H4-Lys^{16} (Abcam, Inc.).

Nuclear factor-κB-luciferase reporter assay. Cells were transfected with a nuclear factor-κB (NF-κB)-luciferase reporter construct alone or with GPR109A cDNA. Twenty-four hours later, cells were pretreated with GPR109A ligands for 4 h and then treated with lipopolysaccharide (LPS; 100 ng/mL) and GPR109A ligands for an additional 4 h. Cells were then lysed, and the lysates used for luciferase activity. NF-κB-luciferase reporter assay was also done with colon tissues obtained from transgenic mice carrying the NF-κB-luciferase transgene under the control of β-actin promoter. Mice were euthanized by CO_2, and colons were removed and everted. Tissue slices (~10 mg wet weight) were used for incubation with LPS with or without pretreatment with GPR109A ligands. Tissues were then homogenized, and the lysates used for measurement of luciferase activity.

Results

Expression of GPR109 in mouse and human colons. We first investigated the expression of GPR109A in mouse intestinal tract and human colon. GPR109A mRNA was detected all through the intestinal tract in mouse (Fig. 1*A*). The expression was lowest in the proximal parts of the intestinal tract and highest in the distal parts. Human colon expressed both isoforms, GPR109A and GPR109B. Two human normal colon cell lines, NCM460 and CCD841, also expressed GPR109A and GPR109B (Fig. 1*B*). Expression of GPR109A, analyzed by immunohistochemistry, was evident

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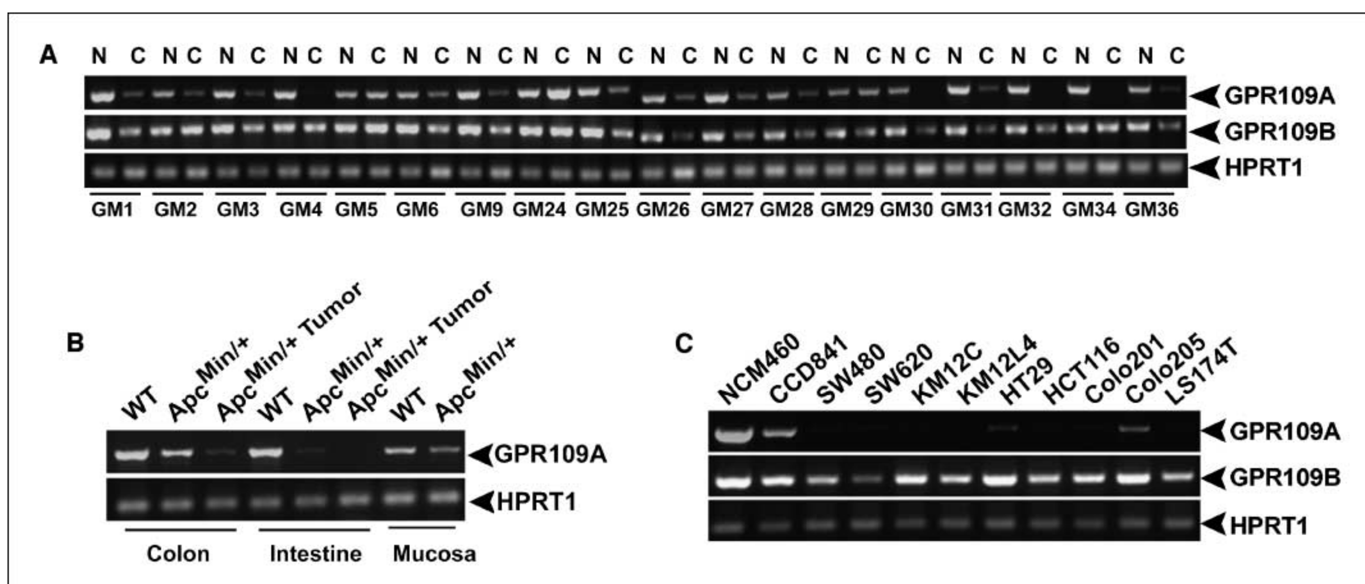

Figure 2. Silencing of *GPR109A* in colon cancer. *A,* expression of *GPR109A* and *GPR109B* in normal colon and paired colon cancer tissues from 18 patients as assessed by RT-PCR. *B,* expression of *GPR109A* in the colon and intestine of wild-type and $Apc^{Min/+}$ mice. In $Apc^{Min/+}$ mice, the tissues were collected from tumor sites and also from areas with no tumor. *C,* comparison of expression of *GPR109A* and *GPR109B* by RT-PCR between normal colon cell lines (*NCM460* and *CCD841*) and cancer colon cell lines.

in small intestine (jejunum and ileum) as well as in large intestine in mouse (Fig. 1*C*). The expression was restricted to the lumen-facing apical membrane of intestinal/colonic epithelial cells. The immunopositive signals were not detected when antigen-neutralized primary antibody was used. Human colonic biopsies were examined for expression of GPR109A and GPR109B using respective antibodies (Fig. 1*D*). Human colon expressed both isoforms of GPR109. The expression of GPR109A as well as of GPR109B in human colon was restricted to the apical membrane.

Butyrate as a ligand for GPR109A. CCD841 cells were used to study nicotinate binding and interaction of butyrate with GPR109A. Specific binding of nicotinate was detected with membranes prepared from these cells; the binding was inhibited by butyrate (10 mmol/L; Supplementary Fig. S1*A*). The binding of nicotinate was of high affinity (K_d, 245 ± 32 nmol/L; Supplementary Fig. S1*B*). The function of butyrate as a GPR109A agonist was investigated using a heterologous expression system in which the coupling of pertussis toxin–sensitive G_i proteins to the receptor was monitored with the activity of inwardly rectifying potassium (GIRK) channels as the readout. GIRK channels open in response to activation of pertussis toxin–sensitive G proteins (e.g., G_i and G_o; ref. 35). We used as a positive control the ability of ectopically expressed adenosine receptor A1R to activate GIRK channels in the presence of adenosine. Expression of GPR109A in HEK293 cells allowed activation of GIRK channels in the presence of nicotinate and butyrate (Supplementary Fig. S1*C* and *D*). In cells transfected with vector alone, nicotinate did not activate the channel. The involvement of GPR109A in the process was further confirmed by the effective blockade of nicotinate-induced activation of the channel by pertussis toxin. These results show that butyrate functions as an agonist for GPR109A.

Relevance of GPR109A to colon cancer. To determine the relevance of GPR109A to the tumor-suppressive effects of butyrate in colon, we first examined the expression of GPR109A in normal colon and in colon cancer in humans. The receptor expression was decreased in a majority of primary colon cancer samples compared with corresponding normal colon samples (the decrease in mRNA levels was evident in 15 of 18 paired samples; Fig. 2*A*). A pairwise comparison (cancer versus normal) with all 18 paired samples showed that the decrease in expression in cancer tissues was 83 ± 5% ($P < 0.001$). The levels of GPR109B mRNA were also reduced in colon cancer, but the decrease was much smaller (34 ± 8%; $P < 0.05$). We also examined the expression of GPR109A in a mouse model of intestinal/colon cancer ($Apc^{Min/+}$ mouse; Fig. 2*B*). The expression of the receptor mRNA was evident in colon, small intestine, and intestinal mucosal scrapings from wild-type mouse. The expression levels markedly decreased in tumor-bearing regions of colon and intestine from $Apc^{Min/+}$ mouse. Even in regions where there was no evidence of tumor, the expression was reduced significantly in $Apc^{Min/+}$ mouse compared with wild-type mouse. We then monitored the expression levels of GPR109A and GPR109B in normal and cancer colon cell lines of human origin (Fig. 2*C*). The normal cell lines CCD841 and NCM460 expressed the receptor, but the expression was markedly reduced in cancer cell lines (SW480, SW620, KM12C, KM12L4, HT29, HCT116, Colo201, Colo205, and LS174T). The expression of *GPR109B* was not significantly different in cancer cell lines compared with normal cell lines.

Role of DNA methylation in the cancer-associated silencing of *GPR109A* in colon. To determine whether the decrease in the expression of *GPR109A* in colon cancer is due to DNA methylation, we treated normal and cancer colon cell lines with the DNA methylation inhibitor 5′-azacytidine and examined the expression of *GPR109A*. The treatment had no effect on GPR109A mRNA levels in normal cell lines. In contrast, the expression of the gene was induced in cancer cell lines in response to treatment (Supplementary Fig. S2*A*), indicating that DNA methylation is involved in the silencing of the gene in cancer cells. To determine which DNA methyltransferase (DNMT) is responsible for this process, we first evaluated the expression levels of different isoforms of DNMTs in normal and cancer cell lines and also in normal colon and in colon cancer (Supplementary Fig. S2*B* and *C*). We found the expression levels of DNMT1 and DNMT3b to be increased significantly in

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colon cancer cell lines and in primary colon cancer. DNMT1 mRNA levels increased 7.5 ± 0.6-fold ($P < 0.001$) in primary colon cancer compared with normal colon. The corresponding value for DNMT3b was 2.5 ± 0.3 ($P < 0.01$). There was no change in the levels of DNMT3a mRNA in primary colon cancer versus normal colon ($P > 0.05$). These data indicated that DNMT1, DNMT3b, or both might be involved in the silencing of *GPR109A* in colon cancer. We then examined the expression of *GPR109A* in HCT116 cells (a human colon cancer cell line positive for the expression of all three isoforms of DNMT) and in isogenic HCT116 cell lines with targeted deletion of DNMT1 ($DNMT1^{-/-}$), DNMT3b ($DNMT3b^{-/-}$), or both (*DKO*). We found very little expression of *GPR109A* in control HCT116 cells, but the expression was markedly induced in $DNMT1^{-/-}$ cells and *DKO* cells but not in $DNMT3b^{-/-}$ cells. This was evident at the levels of mRNA (Fig. 3*A*) and protein (Fig. 3*B*). This suggests that DNMT1 is most likely responsible for the silencing of *GPR109A* in cancer. In contrast to GPR109A, the levels of GPR109B mRNA remained the same in these cell lines, indicating that the expression of *GPR109B* is not regulated by DNMTs. To further confirm the involvement of DNMT1 in the cancer-associated silencing of *GPR109A*, we used procainamide, a specific inhibitor of DNMT1 (36). Treatment of colon cancer cell lines with this compound induced the expression of the receptor (Fig. 3*C*), supporting the conclusion that DNMT1 mediates the silencing of *GPR109A* in cancer cell lines.

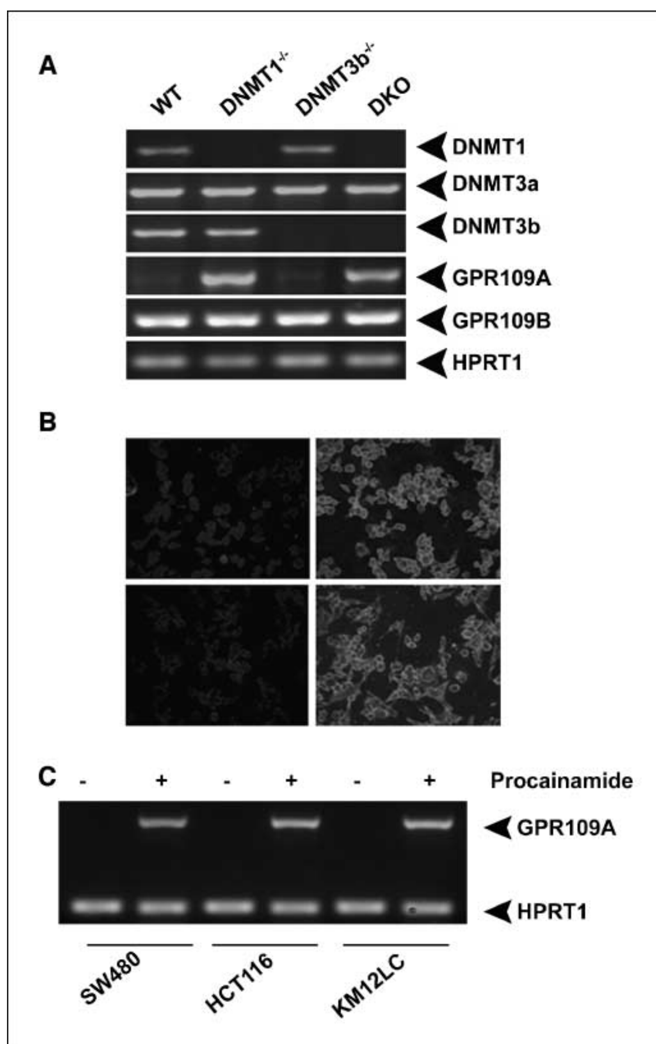

Figure 3. Involvement of DNMT1 in the silencing of *GPR109A*. *A,* analysis of expression of *GPR109A* and *GPR109B* by RT-PCR in the human colon cancer cell line HCT116, which expresses all three isoforms of DNMT (*WT*), and in isogenic HCT116 cell lines with targeted deletion of *DNMT1* ($DNMT1^{-/-}$), *DNMT3b* ($DNMT3b^{-/-}$), or both (*DKO*). *B,* immunocytochemistry for GPR109A protein in WT, $DNMT1^{-/-}$, $DNMT3b^{-/-}$, and DKO cells. *C,* effect of procainamide, a specific inhibitor of DNMT1, on *GPR109A* expression in colon cancer cell lines.

Role of GPR109A in the tumor-suppressive effects of butyrate. To determine whether the butyrate receptor GPR109A has any role in the tumor-suppressive actions of butyrate, we selected CCD841 cells and KM12L4 cells as representatives of a normal colon cell line and a colon cancer cell line, respectively. CCD841 cells constitutively express the receptor. Exposure of these cells to GPR109A ligands butyrate or nicotinate did not have any effect on these cells (Fig. 4*A*). This was true even when the receptor was overexpressed by transfection with an expression vector. KM12L4 cells, being a cancer cell line, do not express the receptor. Accordingly, exposure of these cells to the receptor ligands butyrate or nicotinate did not have any effect (Fig. 4*B*). However, when the receptor was expressed ectopically, exposure of the cells to butyrate or nicotinate induced apoptosis (Fig. 4*B*). The GPR109A/nicotinate–induced apoptosis in KM12L4 cells was associated with activation of caspases (Fig. 4*C*). In addition, the expression of various antiapoptotic genes (*Bcl-2, Bcl-W, Bcl-xL*, and *Bfl-1*) was decreased and that of various proapoptotic genes (*FAS-L, FAS-R, FADD*, and *TNF-R1*) was increased in association with GPR109A/nicotinate–induced apoptosis (Supplementary Fig. S3). Furthermore, the expression of *cyclin D1* was decreased and the expression of *PTEN, PPAR*γ, and *Foxo3A* was increased with GPR109A/nicotinate. Another interesting finding was the subunit switching for phosphatidylinositol 3-kinase in association with GPR109A/nicotinate–induced apoptosis. The expression of *p55*α was increased whereas the expression of *p85*α was decreased. Butyrate can diffuse into mammalian cells to some extent whereas nicotinate cannot. Therefore, to eliminate any potential confusion in the interpretation of the data, we used nicotinate rather than butyrate as the receptor ligand in these experiments.

Noninvolvement of HDAC inhibition in cancer cell apoptosis induced by GPR109A activation. Butyrate is an inhibitor of HDAC. We have previously shown that butyrate induces apoptosis in colon cancer cell lines if SLC5A8, a butyrate transporter, is expressed in these cells and that the process is associated with inhibition of HDACs (20). In the present study, we showed that butyrate induces apoptosis in cancer cells by activation of GPR109A on the cell surface. To determine whether the apoptosis induced in colon cancer cells by GPR109A activation involved HDAC inhibition, we measured HDAC activity in CCD841 and KM12L4 cells under various experimental conditions (Supplementary Fig. S4). HDAC activity was significantly lower in CCD841 cells than in KM12L4 cells, showing that cancer cells have higher HDAC activity. The levels of HDAC activity did not change in CCD841 cells irrespective of whether or not GPR109A was expressed ectopically in these cells or whether or not these cells were exposed to nicotinate (Supplementary Fig. S4*A*). The same was true in KM12L4 cells. Importantly, there was no change in HDAC activity in these cells even after transfection with a GPR109A expression vector followed by treatment with nicotinate (Supplementary Fig. S4 *B*). However, apoptosis was induced in these cells under these conditions. These HDAC

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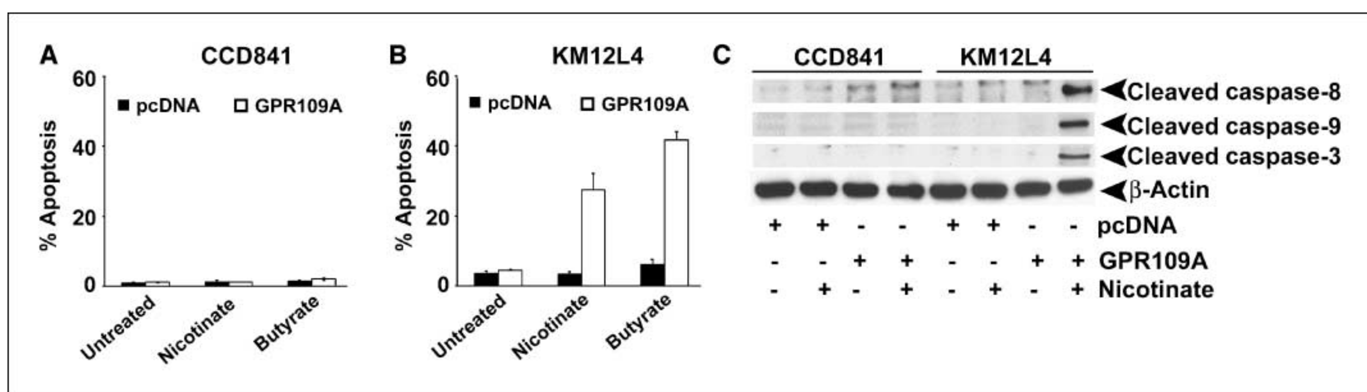

Figure 4. Induction of apoptosis in colon cancer cells by GPR109A ligands. *A,* the normal colon cell line CCD841 was transfected with vector or human GPR109A cDNA, and then treated with or without nicotinate (1 mmol/L) or butyrate (1 mmol/L) for 48 h. Cells were then used for analysis of apoptosis by FACS. *B,* the colon cancer cell line KM12L4 was transfected with vector or human GPR109A cDNA, and then treated with or without nicotinate (1 mmol/L) or butyrate (1 mmol/L) for 48 h. Cells were then used for analysis of apoptosis by FACS. *C,* the cell lysates from the experiments described in *A* and *B* were used to monitor caspase activation by Western blot with antibodies specific for cleaved fragments of caspases.

activity data were confirmed by monitoring the acetylation status of histone H3, histone H4, histone H4-Lys^{12}, and histone H4-Lys^{16} (Supplementary Fig. S4*C*).

Blockade of NF-κB by GPR109A ligands in colon. Normal colonic epithelium expresses the toll-like receptor TLR4, which functions as a receptor for bacterial LPS (37). Intriguingly, there is no undue inflammation of the colonic epithelium under normal conditions in the presence of bacteria in the colon. LPS is known to activate NF-κB signaling through TLR4. Because NF-κB is proinflammatory and protumorigenic, we investigated the relevance of GPR109A and butyrate to LPS-induced NF-κB activation in colon cells. The normal colon cell line CCD841 constitutively expressed GPR109A and TLR4 (data not shown). When transfected with a NF-κB-luciferase reporter, the expression of luciferase in these cells was induced by LPS treatment, but pretreatment with butyrate completely abolished this induction (Fig. 5*A*). This effect was reproduced with nicotinate and acifran, two other agonists of GPR109A. The colon cancer cell line KM12L4 expressed TLR4 but not GPR109A (data not shown). LPS induced luciferase expression in these cells after transfection with the reporter, but GPR109A ligands had no effect on this induction (Fig. 5*B*). This was expected because of the absence of GPR109A. However, with ectopic expression of GPR109A, the induction of luciferase by LPS in this cell line was significantly blocked by GPR109A ligands ($P < 0.001$). These results were corroborated by studies with HCT116 cells, which did not express *GPR109A*, and *DNMT1*$^{-/-}$ isogenic HCT116 cells, which expressed the receptor (Fig. 5*C*). To determine whether these findings are reproducible in normal colon, we used colon tissues from a transgenic mouse that carries the NF-κB-luciferase reporter gene under the control of β-actin promoter. Because normal colon constitutively expresses *GPR109A*, we directly tested the effects of GPR109A ligands on LPS-induced activation of NF-κB reporter in these tissues. Butyrate and other GPR109A ligands were able to block LPS-induced activation of NF-κB in normal colon (Supplementary Fig. S5). Interestingly, activation of GPR109A in normal colon blocked not only LPS-induced activation of NF-κB but also the basal activity of NF-κB.

Discussion

Gut bacteria play a critical role in the prevention of colon cancer and inflammatory bowel disease, but the molecular mechanisms involved in the process are not well understood. The short-chain fatty acids generated by bacterial fermentation of dietary fiber and unabsorbed carbohydrates are believed to be responsible for these effects. Epidemiologic studies indicate that increased intake of fiber in the diet is linked to decreased risks of colon cancer and inflammatory bowel disease (8). Among the short-chain fatty acids produced by the bacteria in colon, butyrate is unique in that it is an inhibitor of HDACs (10, 11). Butyrate induces differentiation in normal intestinal and colonic epithelial cells but causes apoptosis in colon cancer cells (4–7). The ability of butyrate to inhibit HDACs inside the cells provides a molecular mechanism for these effects. This intracellular action of butyrate requires a mechanism for the entry of butyrate into cells. SLC5A8 was recently identified as a Na^+-coupled high-affinity transporter for butyrate; the ability of the transporter to mediate the concentrative entry of butyrate into colon cells offers a mechanism for the tumor-suppressive effect of this bacterial metabolite (21–23).

The present studies unravel a novel mode of action of butyrate in colon, involving the cell surface G-protein–coupled receptor GPR109A. This receptor is expressed in normal colon on the lumen-facing apical membrane of colonic epithelial cells where it has access to luminal contents. Butyrate serves as a ligand for the receptor. Our studies show that this receptor functions as a tumor suppressor in colon. *GPR109A* is expressed in normal colon but is silenced in colon cancer. This phenomenon is also seen in colon cell lines; normal cell lines express the receptor whereas cancer cell lines do not. Activation of the receptor in normal colon cells does not induce cell death. However, when the receptor is expressed ectopically in colon cancer cells, activation of the receptor with butyrate or other ligands leads to apoptosis. The cancer-associated silencing of *GPR109A* occurs via DNA methylation. DNMT1 is responsible for this process. It is not known at present whether *GPR109A* is the direct target for DNMT1 or whether the silencing of *GPR109A* occurs indirectly through some other intermediary gene products. The cell death in colon cancer cells induced by GPR109A activation does not involve inhibition of HDACs. Thus, the bacterial fermentation product butyrate causes cell death in colon cancer cells by two independent but complementary mechanisms: one through SLC5A8-mediated entry of butyrate into cells with subsequent inhibition of HDACs, and the second through GPR109A independent of HDACs. The signaling pathways responsible for GPR109A/butyrate–induced cell death in cancer cells remain to be

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identified. Inhibitors of DNA methylation are currently being evaluated for their utility in cancer chemotherapy (38); unfortunately, clinical trials have shown encouraging results against leukemia but not against solid tumors such as colon cancer. Because our studies show that inhibition of DNA methylation in colon cancer cells induces *GPR109A* expression and that activation of the receptor causes tumor cell–specific apoptosis, the efficacy of DNA methylation inhibitors in the treatment of colon cancer might be enhanced by cotreatment with GPR109A ligands such as nicotinate.

The present studies show that GPR109A functions not only as a tumor suppressor but also as a blocker of LPS-induced NF-κB activation. The NF-κB signaling pathway plays a critical role in colonic inflammation as well as in inflammation-induced cancer (39). Our present findings suggest that butyrate mediates the protective effects of gut bacteria against inflammatory bowel disease by serving as a ligand for GPR109A. This is supported by a recent study, which showed a significant decrease in the number of butyrate-producing bacteria in the colon of patients with ulcerative colitis compared with normal controls (40). Chronic inflammation of the colon as seen in ulcerative colitis is a risk factor for colon cancer; therefore, GPR109A ligands may have potential as therapeutic agents in the treatment of inflammatory bowel disease and colon cancer.

The expression of GPR109B is altered only slightly in colon cancer. Although GPR109B has high homology to GPR109A in primary structure, the two isoforms have marked differences in ligand specificity. GPR109B exhibits drastically reduced affinity than GPR109A for nicotinate and butyrate (28). This suggests that, unlike GPR109A, GPR109B does not function as a butyrate receptor in colon. Because GPR109B is not likely to mediate the biological

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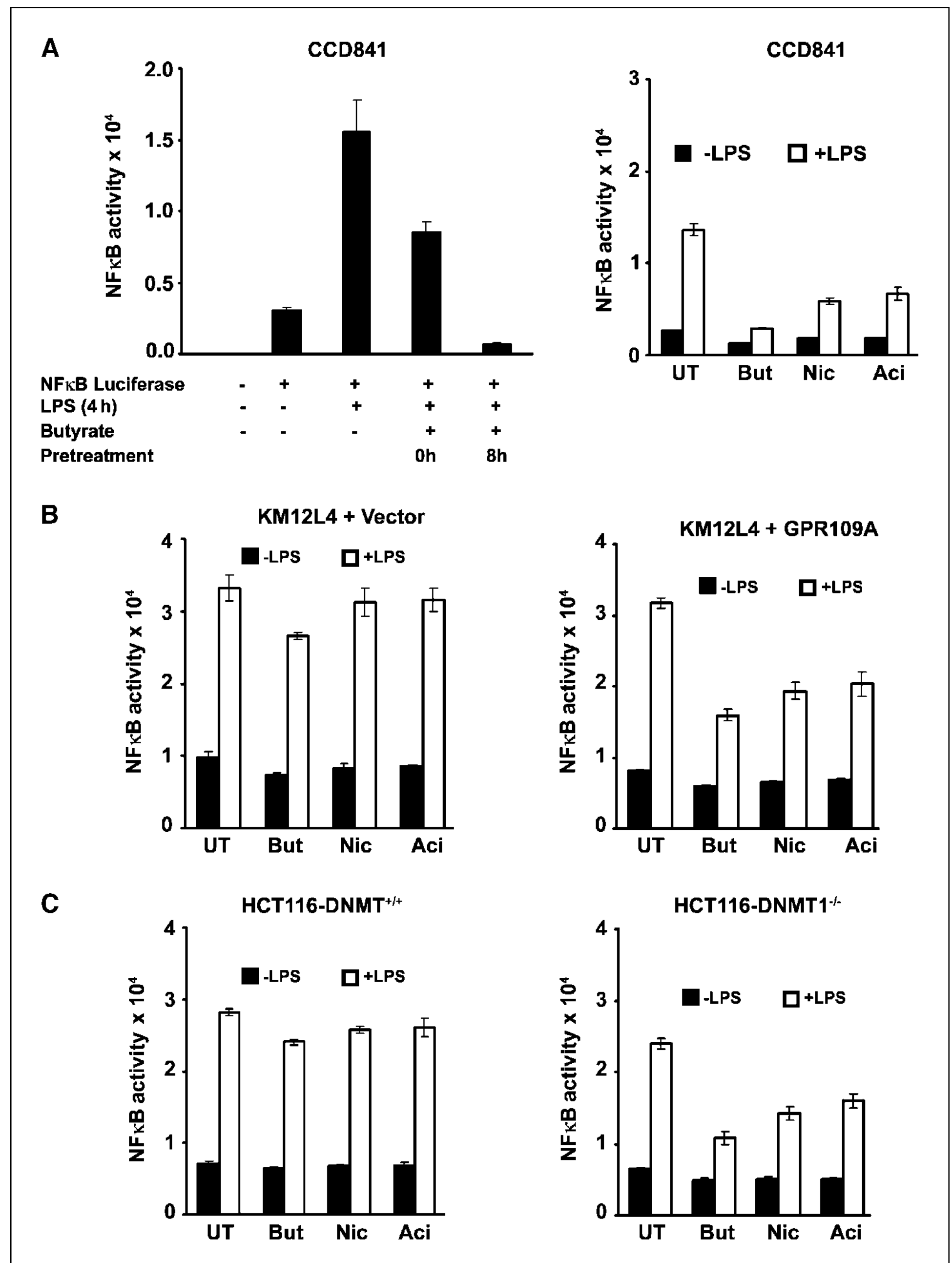

Figure 5. Blockade of LPS-induced NF-κB activation by GPR109A in the normal colon cell line CCD841 and in the colon cancer cell lines KM12L4 and HCT116. *A,* CCD841 cells were first transfected with a NF-κB-luciferase reporter construct. Twenty-four hours later, cells were treated with LPS (100 ng/mL) for 4 h with or without pretreatment with butyrate (*But*; 1 mmol/L), nicotinate (*Nic*; 1 mmol/L), or acifran (*Aci*; 0.25 mmol/L) for 4 h. The ligands were present for an additional 4 h during treatment with LPS. LPS-induced activation of NF-κB was monitored by measuring the activity of luciferase as a reporter. *UT,* no treatment with GPR109A ligands. *B,* KM12L4 cells were transfected with a NF-κB-luciferase reporter together with either vector or GPR109A cDNA. Twenty-four hours later, cells were treated with LPS (100 ng/mL) for 4 h with or without pretreatment with butyrate (mmol/L), nicotinate (1 mmol/L), and acifran (0.25 mmol/L) for 4 h. In addition to the pretreatment, the ligands were present also during LPS treatment. LPS-induced activation of NF-κB was monitored by measuring the activity of luciferase as a reporter. *C,* HCT116 cells (*DNMT*$^{+/+}$ and *DNMT1*$^{-/-}$) were transfected with a NF-κB-luciferase reporter construct. Twenty-four hours later, cells were treated with LPS (100 ng/mL) for 4 h with or without pretreatment with butyrate (1 mmol/L), nicotinate (1 mmol/L), or acifran (0.25 mmol/L) for 4 h. The ligands were present also during LPS treatment. LPS-induced activation of NF-κB was monitored by measuring the activity of luciferase.

effects of butyrate, this isoform is not targeted for silencing in colon cancer. Interestingly, medium-chain fatty acids such as heptanoate and octanoate activate GPR109B; however, these fatty acids are not generated at significant concentrations in colonic lumen by bacterial fermentation. Because GPR109B is expressed almost at normal levels in colon cancer, it might be useful to evaluate in future studies the potential of this receptor as a drug target for treatment of colon cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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